REPOSITIONING OF DRUGS FOR NOVEL USES AND UNDERSTANDING THE MOLECULAR MECHANISM OF DRUG TOXICITY BY CHEMICAL PROTEOMIC APPROACH

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CERTIFICATE

This is to certify that the work presented in the thesis entitled "**Repositioning Of Drugs For Novel Uses And Understanding The Molecular Mechanism Of Drug Toxicity By Chemical Proteomic Approach**" submitted by **Suresh K.K.** was carried out by the candidate at National Chemical Laboratory, Pune under my supervision. Such materials as obtained from other sources have been duly acknowledged in the thesis.

(Dr. Mahesh J. Kulkarni)
Research Guide

April 2014

CANDIDATE'S DECLARATION

I hereby declare that the thesis entitled "**Repositioning Of Drugs For Novel Uses And Understanding The Molecular Mechanism Of Drug Toxicity By Chemical Proteomic Approach**" submitted for the degree of Doctor of Philosophy in Biotechnology to the University of Pune has not been submitted by me to any other university or institution. This work was carried out at CSIR-National Chemical Laboratory, Pune, India.

Suresh K.K. **April 2014**
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Dedicated to…..

My family, who encouraged me…..

"And, when you want something,
all the universe conspires in
helping you to achieve it."

— Paulo Coelho,

The Alchemist

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Abbreviations

ACN	-	Acetonitrile
CBB	-	Coommassie Brilliant Blue
CHAPS	-	3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate
CBB-R250	-	Coommassie Brilliant Blue R250
Da, kDa	-	Dalton, Kilodalton
DAB	-	2,4-Diaminobutyric Acid
DTT	-	1,4-dithio-D-threitol
EDTA	-	Ethylenediamminetetraacetate
ESI MS	-	Electro spray ionisation mass spectrometry
g	-	Relative centrifugal force
g, µg, ng	-	Gram, milligram, microgram, nanogram
GFP	-	Glu-fibrinopeptide B
HbA1c	-	Glycated haemoglobin
HDMS	-	High definition mass spectrometry
HSA	-	Human serum albumin
IAA	-	Iodoacetamide
IgG	-	Immunoglobin G
IPG	-	Immobilized pH gradient
MALDI MS	-	Matrix associated laser desorption ionisation MS
MS^E	-	MS at elevated energy
PLGS	-	Protein Lynx Global Server
PVDF	-	Polyvinylidene fluride
STZ	-	Streptozotocin
TBS	-	Tris buffered saline
V, kV	-	Volt, kilovolt
2DE	-	Two dimensional gel electrophoresis
HYD	-	Hydralazine
AMG	-	Aminoguanidine

HPLC	-	High pressure liquid chromatography
min	-	Minutes
mL	-	Millilitre
ASP	-	Aspirin
GATI	-	Gatifloxacin
DIAB	-	Diabetic
AGEs	-	Advanced Glycation Endproducts
MIC	-	Minimum inhibitory concentration
TPP	-	Triphenylphosphine
GSAR	-	Glucose Sensitive Amino acid Residue
BUN	-	Blood Urea Nitrogen
NBT	-	Nitroblue tetrazolium
ELISA	-	Enzyme-linked Immunosorbent Assay

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Abstract

Drug discovery is an extensive process involving target identification, small molecule screening, optimization, preclinical and clinical trials and finally to market. The whole process is time consuming, expensive and has a high risk of failure, as several winning candidates may have to be dropped for undesired bio-distribution and toxicity shown by them. The number of new drugs released in the market are decreasing and there is acute pressure on the pharmaceutical industry to increase the number of candidate drugs in the late stage pipeline. Many times the toxicity is shown long after the approval of the drug that leads to its withdrawal from market e.g. cerivastatin. Therefore, a new concept called "drug repositioning" is being emerged. Drug repositioning is the process of finding new uses outside the scope of the original medical indication for existing drugs. There are two approaches used here- 1) finding new indications, 2) formulating new delivery system. The thesis focus was on the first approach. This approach mainly involves identifying a new interacting protein(s), as most of the drugs act or cause toxicity through binding to different proteins. Thus studying drug- protein interaction becomes important for finding new drug targets and elucidating toxicity mechanisms.

Comparative and Chemical Proteomic Approaches Reveals the Mechanism of Gatifloxacin Induced Abnormalities of Glucose Metabolism

Gatifloxacin has been associated with increased risks of hypoglycaemic and hyperglycaemic side effects. In order to understand the molecular mechanism of gatifloxacin induced deregulation of glucose metabolism, a combination of comparative and chemical proteomic approaches were employed using *Saccharomyces cerevisiae* as a model system. Differential protein expression studies using two dimensional electrophoresis and mass spectrometry revealed that gatifloxacin deregulates the expression of key enzymes involved in glucose metabolism. Furthermore, affinity chromatography and LC-MS^E analysis led to identification of enolase, as one of the key gatifloxacin binding proteins. Fluorescence spectrometric studies confirmed that the gatifloxacin indeed binds to enolase. Role of enolase in regulation of gatifloxacin induced dysglycaemic effect is discussed.

Proteome Wide Reduction of AGE Modification in Diabetic Mice by Hydralazine Mediated Transglycation

The non-enzymatic reaction between glucose and protein can be chemically reversed by transglycation. Here we report the transglycation activity of hydralazine using a newly developed MALDI-TOF-MS based assay. Hydralazine mediated transglycation of HbA1c, plasma proteins and kidney proteins was demonstrated in streptozotocin (STZ) induced diabetic mice, as

evidenced by decrease in protein glycation, as well as presence of hydralazine-glucose conjugate in urine of diabetic mice treated with hydralazine. Hydralazine down regulated the expression of Receptor for Advanced Glycation End products (RAGE), NADPH oxidase (NOX), and super oxide dismutase (SOD). These findings will provide a new dimension for developing intervention strategies for the treatment of glycation associated diseases such as diabetes complications, atherosclerosis, and aging.

Inhibition of Advanced Glycation End Products Formation by Aspirin Mediated Pre-acetylation of Proteins

Glycation is implicated in many disease including aging, diabetic complications, neurodegenerative diseases, atherosclerosis etc. Inhibition or reduction of glycation has been suggested to be one of the intervention strategies in the treatment and management of diabetes. Small molecules such as nucleophilic amines like amino acids, guanidines and hydrazines have the capability to competitively inhibit the glycation. In addition certain molecules like acetyl salicylic acid (ASA or Aspirin) inhibit glycation by acetylating lysine residue and thus protecting those residues from glycation. Here we have addressed whether pre-acetylation of proteins can protect them from glycation. *In vitro,* proteins were pre-acetylated by aspirin and then the effect of glycation was studied. It was observed that pre-acetylation protects proteins from AGE formation, change in secondary structure, protein cross-linking and aggregation. Additionally aspirin mediate pre-acetylation reduces HbA1c and AGE formation in the Streptozotocin induced diabetic mice. Hence pre-acetylating

agents like aspirin have an additional therapeutic efficacy of reducing AGE levels. The identification of these novel acetylating agents represents a new area for drug discovery process.

Chapter 1

Drug Repositioning: Opportunities and Challenges

1.1 Introduction

Drug discovery is the process of identification of biologically active small molecules against different disease conditions. The testing of large collections of small molecules for a specific pharmacological effect is the classical route to discover novel lead compounds which subsequently serve as templates for further optimization. Classical drug discovery starts from the identification of disease target, lead compound identification and optimization, ADMET studies and finally to market. Developing a Single molecule may take $10-17$ years and the success rate can be as low as 0.01%[1, 2, 3, 4]. The quantum leap in the research and development in this area can be attributed primarily to the extraordinary progress that has been made in the areas like genomics, proteomics, lipidomics, metabolomics and information technologies. The advent of improved assay techniques and high-performance laboratory automation dramatically changed the pharmacological screening

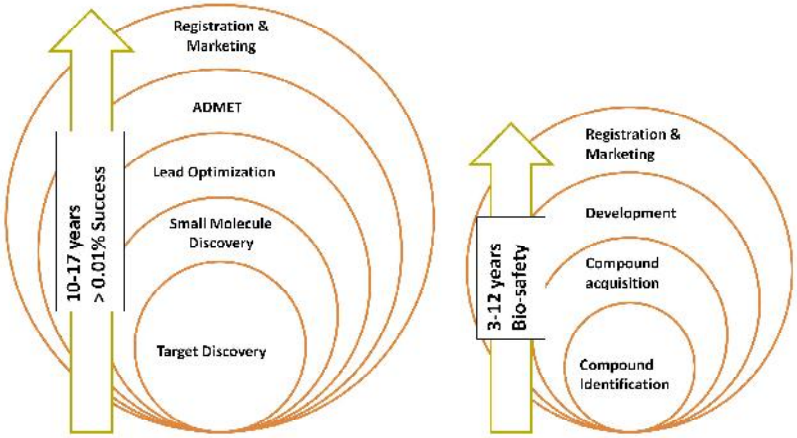

Figure 1.1: **Drug Discovery Pipeline: Classical Vs Repositioning.** Classical drug discovery may take 10-17 years to bring a drug into the market with high risk of failure. Drug repositioning offers assured bio-safety with reduced time and costs

process. The global annual budgets of R&D became $ 130 billion with fewer new drugs[1]. The numbers of new drugs or New Molecular Entities (NME) released in the market are decreased and there is acute pressure on the R&D circle to increase the number of candidate drugs in the late stage pipeline. Considering the large amount of investment that has been put recently in drug development only 23 drugs were approved in the year 2010, which is drastically less when compared to the drugs approved (53 drugs) a decade ago. [1]

These NMEs have to go through a number of pharmacokinetic and toxicity studies for their release into market. Molecules with potential drug like activities are evaluated simultaneously for their toxicity effects in cell and animal models. After a strenuous and systematic evaluation of drug activity and other properties, several drug like molecules may have to be dropped because of undesired bio-distribution and toxicity. Recently, in the

year 2007, 103 NMEs were approved by FDA, out of which 91 NMEs showed failure in phase 3 trials. A classical example of such a kind was AstraZeneca's NXY-059[5] which gave very promising results in the first clinical trial for the amelioration of stroke-induced injury and disability, but failed in the second phase trials. Many times the toxicity is shown long after the approval of the drug that lead to its withdrawal from market e.g. Baycol (cerivastatin) of Bayer AG[6]. Baycol, a cholesterol-lowering drug was withdrawn from the market in 2001 as large number of patients suffered from the rhabdomyolysis, a muscle-weakening disorder. When toxicity is unacceptable at the levels of administered dosage, these molecules are discontinued in spite of their good activity, bio-distribution and pharmacokinetic properties. Therefore, a new concept called "drug repositioning" is being emerged in the pharmaceutical R&D circle. Drug repositioning is the process of finding new uses outside the scope of the original medical indication for which the drug is prescribed [1, 4]. A repositioned drug can go directly to preclinical testing and clinical trials and save the initial $6-9$ years essentially needed for the development of a new drug, with reduced risk and costs[7]. Such new uses have been identified, normally by serendipitous treatment or unexpected side effects observed during clinical trials[8, 1]. Drug repositioning need to be more scientific and rational to identify new uses for existing drugs.

1.2 Approaches for Drug repositioning

Drug repositioning is achieved by understanding of molecular mechanisms of drug action and by identification of the interacting proteins of the drug[9]. In many cases, molecular mechanism of drug action is poorly understood or completely unknown. The two approaches that are used generally to en-

hance the "usability of a drug" are: 1) finding new indications or therapeutic window [9], 2) formulating new delivery system either to enhance the bio-availability or to avoid drug induced toxicity[10]. Here we focus on the first approach, that is to understand the mechanism of drug action that may leads to identification of new therapeutic window for a drug. The drug action can be observed by identification of drug targets and their specific interactions, drug induced change in expression of a specific gene and the associated pathways and change in disease phenotypes. Researchers all over the world are utilizing both, data driven approaches[11] as well as blinded target or assay based approaches[12] to solve the problem. The different approaches used for repositioning range from small molecular modeling[13, 14], gene or protein expression profiling[15, 16], chemical proteomics[17, 18], phenotypic screening[12], clinical observations[19], side effect analysis[20], and to text mining[21, 22]. Therefore researchers utilize the complete "drug action spectrum", i.e. the bio active molecules, to its interacting partners, to differential biological pathways, and finally, to changed phenotypes. The data driven approaches (DDA) utilizes the development in chemoinformatics, bioinformatics as well the development of techniques like genomics, proteomics and metabolomics. DDA are mostly integrative discovery based approaches and further validated by conventional drug discovery approaches.

1.2.1 From small molecule space

Bioactive small molecules are a vast spectrum of chemical space; need to be explored with new innovations on data mining. The main approaches are direct chemical similarity scoring[23], new pharmacophore identification[24], Fragment based drug design[25, 27] and reverse docking[14, 26, 28]. Direct chemical similarity scoring approach begins from the hypothesis that similar

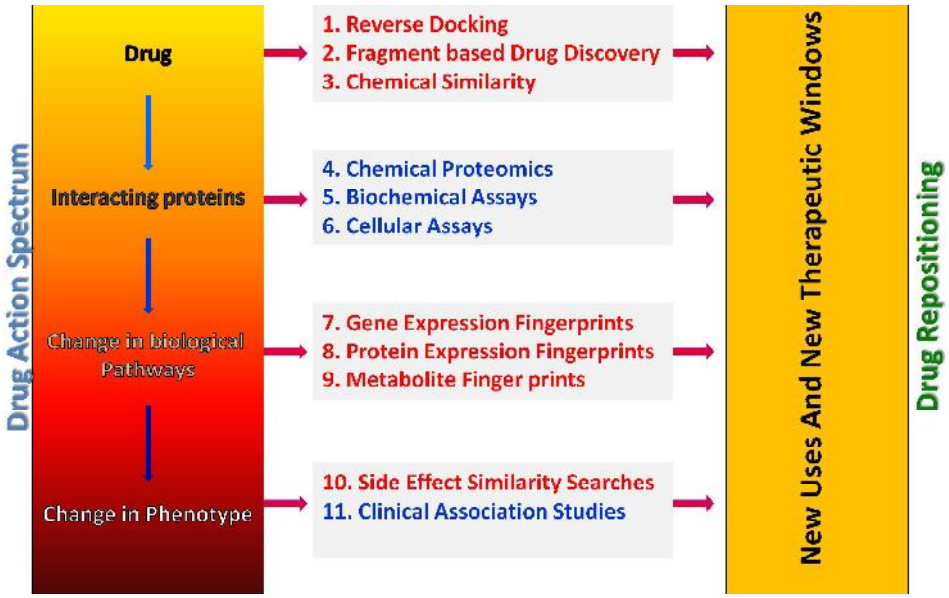

Figure 1.2: **Drug action spectrum and different approaches for drug repositioning.** Drug repositioning have different intergrative approaches like fragment based drug discovery, Docking, Gene expression fingerprints, Side effect similarity searches, etc. This is made only possible by the progress in areas like genomics, proteomics, lipidomics, metabolomics and information technologies

compounds have similar targets and can be used against similar disease physiology. This approach was further improved by calculating the similarities in pharmacophore and fragment based drug design. To identify the new uses Keiser *et al.* relates the topology of 3665 FDA approved small molecules to known 1400 protein targets and proved the serotonergic receptor interaction of the drug N,N-dimethyltryptamine[23]. The Fragment based drug design (FBDD), is an alternative to high throughput screening, where in identifying different low molecular weight molecules that are able to interact with a target at different binding sites. There after they may get combined or grown into high affinity ligands. This approach leads to the identification of new inhibitors for Cyclophilin A,B and D[25, 27]. Reverse docking is the *in silico* approach to identify the multiple protein or target interaction of a small molecule[14, 26, 28]. Here, small molecules are docked against different protein structures. Multiple Ligand Simultaneous Docking (MLSD) is one of the technique to identify celecoxib as a novel inhibitor of STAT3. But this method is limited with the lack of good drug and target scaffold databases.

1.2.2 From drug-target interactions

Deciphering the drug target interaction is the key to understand mechanism of drug action. The experimental approaches used for identifying the interacting targets are biochemical assays, cell based assays and chemical proteomics approach. In biochemical and cell based assays, the library of small molecules are analyzed against known sets of drug target assays and the lead compounds are further optimized. Chemical proteomics is a discovery approach, where different interacting partners are pull down by affinity approaches [29, 18]. It tries to identify new interacting protein(s) and understand its relevance with respect to different disease physiology, as most of

the drugs act or cause toxicity through binding to different proteins. Such studies can reveal two possibilities of drug action: 1) a same interacting protein is involved in the manifestation of more than one disease or 2) a drug can binds to multiple proteins of different disease conditions called polypharmacology[**?**, 30]. Studies carried out at the Northwestern University support the first possibility, revealing that the entry of the malarial parasite Plasmodium falciparum into erythrocytes involves signaling through the host β-adrenergic receptor and the heterotrimeric guanine nucleotide binding protein (Gas). Thus β-blockers commonly used to treat hypertension can be used for the treatment of malaria[31]. The other possibility is that a drug can binds to multiple proteins and offers protection against different diseases like Aspirin. It acts as a NSAID by irreversibly inhibiting COX-1 and modifying the activity of COX-2[**?**]. It also induces the expression of SSAT (spermidine/spermine N1-acetyltransferase), a catabolic enzyme, in HT29 cells[32], thus can be used for cancer treatment. Likewise, there are number of examples in which a drug has shown multiple activities such as thalidomide[33], colchicines [**?**], etc. Besides identifying their beneficial effects or new targets, chemical proteomic approach can identify the interactions which lead to drug toxicity[34]. If these interactions are studied in detail, exact mechanism of toxicity can be understood, which can further help in redesigning the molecule. If such molecules are redesigned, repertoire of new potent drugs will be available. This redesign, recycle and reuse approach will be extremely beneficial as such drugs which are discontinued or withdrawn from the trials have a good drug like activity. Drug- protein interaction studies need to be carried out to identify new potential off-targets of the drugs as well as to identify other interactions that cause toxicity. These studies may throw light on physiology of the drug activity which is involved in eliciting efficacy

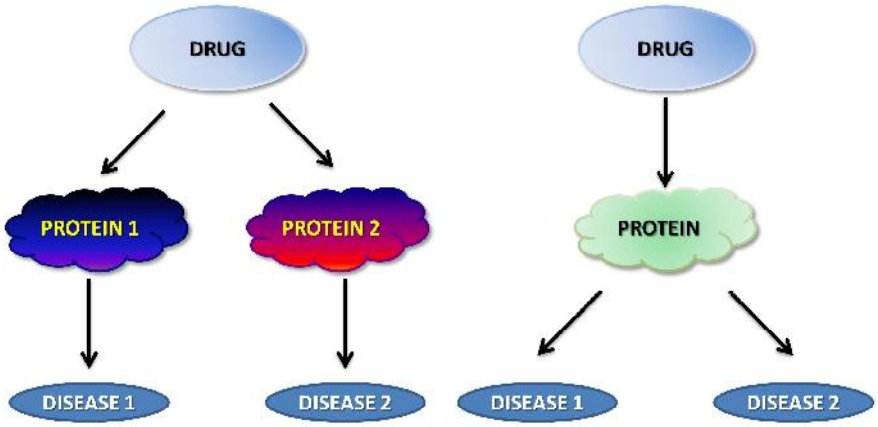

Figure 1.3: **Drug protein interactions: One Drug and many interacting partners or One Interacting Protein with different functions.** Functional diversity of the drug action can be achieved through the interacting partners. This diversity may arise from the drug's interaction with multiple targets or by the epigenetic diversity of the target protein

and toxicity. Many methods have been utilized for the identification of drug-protein interactions. To start with, one has to go from generalized methods to the specific ones. Once the drug-protein interaction is identified, one can go to specific binding studies like fluorescence quenching, surface plasmon resonance, etc. which can be used to validate the interactions. Affinity chromatography was used to identify glycerol-3-phosphate dehydrogenase as a target for Cymelarsan, an arsenical drug used in African trypanosomiasis treatment[35]. The new innovations like quantitative chemical proteomics approach were able to identify as well as quantify the extent of drug target interaction.

1.2.3 From Biological Pathways

Many of the drug targets are not identified yet. Hence drug induced changes in biological pathways can be used as a finger print of its action and comparison of these can elucidate the mechanism as well as gives an opportunity for drug repositioning. These different pathways can be looked at gene, protein, metabolite or system level. Until now 10997 genomes have been sequenced and in that, 2444 of eukaryotes, 20031 of prokaryotes and 3905 of viruses. This helps in better understanding of genes involved in various diseases. Genomes and proteomes being elucidated for several model organisms coupled with constant improvement in understanding of the human genome, the molecular mechanisms of diseases and drug actions can be understood from varied perception. Automated high throughput approaches have helped in developing a holistic understanding of the molecular interactions in disease physiology. The direct relationship between drugs and indications related to gene expression may involve in the primary mechanism of the drug. The DNA microarrays or gene chips are used for the rapid determination of gene expression profiles across the entire genome. Such transcriptome profiling is often useful for the identification of genes that are affected by a given small molecule on a genome-wide level. For examples Marton *et al.*, 1998 used DNA microarrays to identify and validate the drug target of immunosuppressants; FK506, and cyclosporine A[36] . Conserved Anti-coexpressed Gene Clusters (CAGCs) identification is another gene expression analysis, where the disease induced changes in gene expression cluster is identified and look for the drugs that have opposite effect, which can ameliorate the disease physiology[15]. This method helped to identify cimetidine, an antagonist of histamine H2 for effective therapy against lung cancer [16], and the antiepileptic, topiramate can be effective against inflammatory bowel disease [37]. Yoshiya Oda,

et al. used two-dimensional electrophoresis (2-D PAGE) for the identification of drug targets for E7070, an anticancer drug, acts on Cytosolic malate dehydrogenase[38]. Qing-Xi Yue *et al.* in 2008 demonstrated the power of differential expression studies to identify and validate the target protein of ganoderic acid D[39].

Table 1.1: Examples of repositioned drugs and the new indications

Sl. No.	**Drug**	**Mechanism of Action**	**Original indication**	**New indication**
1	Bupropion	Enhancement of noradrenaline function	Depression	Smoking cessation
2	Dapoxetine	Selective Serotonin reuptake inhibitor	Analgesia and Depression	Premature ejaculation
3	Duloxetine	Non-selective Serotonin reuptake inhibitor	Depression Stress	urinary incontinence
4	Fluoxetine	Selective Serotonin reuptake inhibitor	Depression	Premenstrual dysphoria
5	Milnacipran	Non - selective Serotonin reuptake inhibitor	Depression	Fibromyalgia syndrome
6	Sibutramine	Non - selective Serotonin reuptake inhibitor	Depression	Obesity
7	Atomoxetine	Non - selective Serotonin reuptake inhibitor	Parkinson's disease	Attention deficient Hyperactivity Disorder
8	Chlorpromazine	Dopamine Receptor blockade	Antihistamine	Non-sedating tranquillizer

Table 1.1: Continued

9	Galantamine	Acetylcholinestrase inhibition	Polio, Paralysis, and anaesthesia	Alzheimer's disease
10	Lindocaine	Sodium Channel Blockade	Local Anaesthesia	Oral Cortico steroid dependent asthama
11	Ropinirole	Dopamine-2- agonism	Hypertension	Parkinson's disease and idiopathic restless leg syndrome
12	Tofisopam	Unclear	Anxiety related conditions	Irritable bowel syndrome
13	Celecoxib	Cyclooxygenase-2 inhibitor	Osteoarthritis and adult rheumatoid arthritis	Familial adenomatous polyposis, colon and breast cancer
14	Eflornithine	Ornithine decarboxylase inhibition	Anti-infective	Reduction of unwanted facial hair in women
15	Finasteride	5-α-reductase inhibition	Benign Prostatic hyperplasia	Hair loss
16	Mecamylamine	Nicotinic receptor antagonism	Hypertension	Attention deficient Hyperactivity Disorder
17	Mifepristone	Glucocorticoid receptor type II antagonism	Pregnancy termination	Psychotic major depression
18	Minoxidil	β-adrenoceptor blockade	Hypertension	Hair loss
19	Paclitaxel	Attaches polymerization of tubulin	Cancer	Restenosis

Table 1.1: Continued

20	Phentolamine	α-adrenoceptor antagonism	Hypertension	Impaired night vision
21	Raloxifene	SERM	Breast and prostate cancer	Osteoporosis
22	Sidenafil	PDE5 inhibition	Angina	Male erectile dysfunction
23	Tadalafil	PDE5 inhibition	Inflammation and cardiovascular disease	Male erectile dysfunction
24	Thalidomide	TNF-α inhibition	Sedation, nausea and insomnia	Leprosy and multiple myeloma
25	Topiramate	Na Channel Blockade, GABA stimulation	Epilepsy	Obesity
26	Zidovudine	Reverse transcriptase inhibition	Cancer	HIV/AIDS

1.2.4 From Phenotypes

Phenotypic screening is one of the widely used method for screening drugs in the last decade. *In vivo* phenotypic screening has delivered many drugs, often with poor understanding on the drug target or mechanism of action. The target-centric approaches with automated High Through-put Screening resulted in the discovery of many clinical candidates; though successful registration of new drugs has not increased noticeably. Unexpected clinical effects of drugs at different levels like; cell lines, preclinical trials or patients,

have been lead to the identification of novel uses. E.g., Sildenafil, introduced against hypertension has been marketed in the treatment of erectile dysfunction[40]. Systematic drug repositioning approaches based on phenotypic effects like side effect similarity, clinical association studies, etc have an added advantage of clinical safety and prescription reception from physicians. Large scale computational approach utilizes side-effect similarities of drugs to infer shared drug targets. Campillos *et al.* proved these associations by *in vitro* binding assays and cell line assays[20]. The large scale phenotypic associations were utilized further for the cross validation of drug protein interactions. Indeed, drugs with similar side-effect profiles may share therapeutic properties through similar mechanisms of action.

1.3 Genesis and organization of Thesis

Drug repurposing or repositioning offers a cost effective alternative to classical drug discovery. Drug-repositioning has resulted in a variety of innovative amalgamation of computational methods for the identification of new opportunities for old drugs. The comprehensive understanding of these different methods with respect to biological and pharmaceutical knowledge can elucidate the mechanism-of action of drugs. That may provide novel lead generation capabilities. Newer developments in bioinformatics may able to alleviate this problem by large-level integration of available data and elucidation of newer drug action mechanisms. The discovery process can have the advantage of the developments available in chemoinformatics, bioinformatics and systems biology to make full use of known targets, drugs and disease biomarkers or pathways, thus leading to the development of proof-of-concept methods and the design of clinical studies with accelerated timelines. So

Drug repositioning becomes one of the promising areas for pharmaceutical industry in near future, with new means and ends. Thus, we tried to develop methodologies to identify drug targets by chemical proteomics approach and relate its physiological relevance to different diseases. The drug is also repurposed by disease based targeted approaches. Here we developed MALDI-MS based transglycation assay and further proved the drug activity in animal models.

Major objectives of the thesis are as follows

- Identify drug target interactions by chemical proteomic approaches
- Develop assay to screen and identify bioactive small molecules
- Identify new uses for drugs and show its activity in animal models

This thesis is organized and presented in the following manner

Chapter 1: Introduction to drug repositioning: opportunities and challenges.

Chapter 2: Comparative and chemical proteomic approaches reveals the mechanism of gatifloxacin induced abnormalities of glucose metabolism.

Chapter 3: Proteome wide reduction of AGE (Advanced Glycation End-products) modification in diabetic mice by hydralazine mediated transglycation.

Chapter 4: Inhibition of advanced glycation end products formation by aspirin mediated preacetylation of proteins.

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Chapter 2

Proteomic Approaches Reveals the Mechanism of Gatifloxacin Induced Abnormalities of Glucose Metabolism

2.1 Introduction

Gatifloxacin is one of the fourth generation fluoroquinolones used as an antibiotic, which acts by inhibiting bacterial topoisomerase II and DNA gyrase 4 [1]. Although gatifloxacin was withdrawn from the US market, the drug is still being used extensively in other parts of the world for treatment of multidrug-resistant infectious diseases. The drug was withdrawn because of the fact that it exhibits acute hypoglycemic and chronic hyperglycemic side effects [2, 3, 4, 5, 6]. The acute hypoglycemic effect could be due to increased insulin secretion triggered by inhibition of ATP dependent potassium channel in the beta cells of pancreas[7, 8]. Additionally, a recent study

suggests that the gatifloxacin affects gluconeogenesis by inhibiting pyruvate transport to mitochondria[9]. In animal studies, gatifloxacin has been shown to increase epinephrine release when given in higher doses, thus increasing the metabolic rate leading to hypoglycemic condition [10]. In contrast, chronic hyperglycemic effect could be due to the down regulation of glucose transporter 1 (GLUT1) expression in presence of gatifloxacin [11, 12]. Interestingly, in the same study, it was also demonstrated that the gatifloxacin increases the GLUT1 promoter activity and decreases the mRNA levels of the gene suggesting that the drug affects the stability of the GLUT1 mRNA by a mechanism yet to be understood. Gatifloxacin effect on glucose metabolism could be also due to altered protein expression. It is important to understand the influence of gatifloxacin on the regulation of enzymes involved in glucose metabolism. In the present study we report the influence of gatifloxacin on the protein expression with a special emphasis to proteins involved in glucose metabolism using yeast as a model system by combination of comparative [13, 14, 15] and chemical proteomic approaches [16]. Further, we studied the interaction of gatifloxacin with enolase by fluorescence spectrometry. Workflow for the whole study is depicted in the figure 2.1

2.2 Materials and Methods

2.2.1 Materials

YPD Medium for yeast culture was obtained from Hi-media India, BioLyte 3−10 carrier ampholytes and IPG strips were obtained from Bio-Rad, proteomic grade trypsin was from Sigma -Aldrich, and acetonitrile (MS grade) was purchased from Fisher Scientific. Water (18.2 milliΩ) for all experiments was distilled and purified by Milli-Q synthesis (Milli-Q), and all other chem-

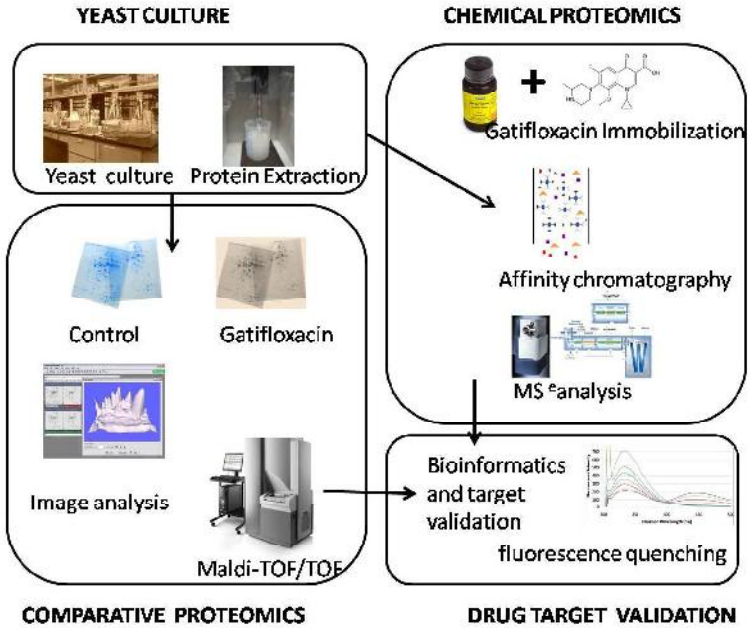

Figure 2.1: **Experimental workflow.** Chemical and comparative proteomics approaches were used to identify the drug targets from *s. cerevisiae.* Further it was validated by fluorescence quenching experiment.

icals were purchased from Sigma-Aldrich. Gatifloxacin was a generous gift from Dr. M.K. Gurjar, Director, Emcure Pharmaceutical Ltd, Pune.

2.2.2 Yeast Culture and Minimum Inhibitory Concentration*(MIC)* of Gatifloxacin

The *Saccharomyces cerevisiae* (NCYC 957) strain was used to study the influence of gatifloxacin on proteins involved in glucose metabolism. Cells were batch-cultured in YPD 2% growth medium at 28°C with a constant rotation at 200 rpm. Minimum Inhibitory concentration (MIC_{50}) of gatifloxacin was determined by growing cells on different concentration of gatifloxacin ranging from 60 mg/L to 250 mg/L for 18 hrs at 28°C [17]. The growth of yeast was spectroscopically monitored at 600 nm.

2.2.3 Estimation of Glucose Utilization by Yeast

Glucose utilization by yeast was analyzed in both control and gatifloxacin treated yeast culture by monitoring the residual glucose with a glucose-oxidase-peroxidase reaction kit (Merck). At different points of time, supernatant of YPD medium was separated from the yeast cells by centrifugation at 4,000 g for 10 min. 10 μl of supernatant was used to analyze glucose.

2.2.4 Protein Extraction

Protein was extracted in buffer containing 7 M Urea, 2 M Thiourea, 4 % CHAPS, 1 % DTT and 2 % general protease inhibitor cocktail (Sigma). Yeast cells were sonicated for 30 sec and the sample was cooled in ice for 2 min and this cycle was repeated thrice. The cell lysate was centrifuged at 10,000 g for 30 min. The supernatant was collected and precipitated with

80% cold acetone containing 10 % TCA and incubated at -20°C for overnight, Precipitate was centrifuged at 10,000 g for 30 min at 4°C and the pellet was resolubilized in rehydration buffer (7 M Urea, 2 M Thiourea, 4 % CHAPS, 1 % DTT and 2 % 3−10 Biolyte Ampholytes from Bio-Rad). The protein concentration was determined by Bradford method.

2.2.5 Two Dimensional Electrophoresis

Isoelectrofocusing (IEF) was carried out on linear narrow range immobilized pH gradients strips of 17 cm (pH 5−8, Bio-Rad) using Protean Isoelectric focusing system (Bio-Rad). The voltage was set to 250 V for 1 h, and then ramped to 10,000 V for 3 h, which was continued at 10,000 V until 72,000 Vh reached. IPG strips were equilibrated with dithiothreitol and iodoacetamide and a second-dimensional electrophoresis was performed by using 12 % SDS-PAGE. Gels were stained with coomassie brilliant blue to visualize the protein spots. Images were acquired using GS 800 densitometer (Bio-Rad) and data analysis was carried out by PDQuest advanced software (Bio-Rad). Protein spots in each gel image were initially detected using the PDQuest spot detection wizard, followed by editing to remove incorrectly detected artifacts. A master image of combined spots from both gels was used for image analysis. Analysis was carried out for three biological replicates. Normalization factor was calculated between different replicates using the total optical density of the gel. The density of each protein was expressed as mean ± SD for three experiments. The statistical significance was established by Students t-test. Differences were considered significant if $P<0.05$.

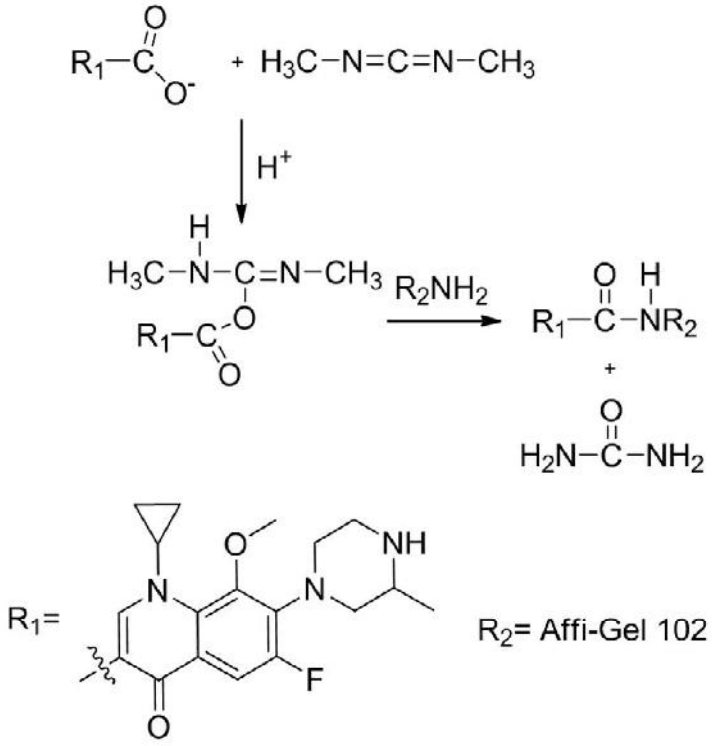

Figure 2.2: **Immobilization of gatifloxacin on to Affigel-102 (Bio-rad) by EDAC coupling.** where R1 is gatifloxacin, R2 is Affigel-102. EDAC carbodiimide coupling reagent helps to immobilize ligands that contain primary or terminal carboxyl groups to the gel.

2.2.6 Affinity Chromatography

An affinity chromatography of gatifloxacin was developed by immobilizing carboxylic group of the drug onto Affigel 102 resin using EDAC coupling as described in Fig. 2.2 for chemical proteomic analysis. The resin (bed volume 1 ml) was packed in a 2.5 ml chromatography column and equilibrated with MilliQ water (pH was adjusted to 4.7−5 with HCl). Gatifloxacin (10 mg) dissolved in 1 ml of water (pH 4.7−5) was applied onto Affigel 102, to which 10mg EDAC coupling reagent was added. The reaction mixture was kept

at room temperature for 4 h. After the reaction, the column was washed with milliQ water (pH 4.7) to remove unbound gatifloxacin. Coupling of gatifloxacin to the resin was monitored spectrometrically by measuring the unbound drug at 287.5 nm, which is the Amax for gatifloxacin. Drug bound Affigel 102 was washed with equilibration buffer containing 250 mM ammonium acetate, 50 mM magnesium chloride, 0.01 % triton X-100 buffer (pH 8.8). Protein lysate (1.5 mg) extracted from yeast in equilibration buffer (250 mM ammonium acetate, 50 mM Magnesium chloride, 0.01 % Triton X-100 buffer pH 8.8) containing 50 μl/ml of general protease inhibitor (Sigma), was incubated with gatifloxacin immobilized on to affigel beads. The column was washed thoroughly to remove unbound protein. Washing was continued till the A280 reading was stabilized. Bound proteins were eluted with gatifloxacin (10 mg/ml) dissolved in ammonium acetate buffer. The eluate containing proteins was concentrated by lyophilization and further digested with trypsin.

2.2.7 Mass Spectrometry Analysis and Protein Identification

The proteins separated on two dimensional electrophoresis gel and digested peptides were analyzed using the Applied Biosystems 4800 MALDI TOF/TOF Analyzer (Applied Biosystems, Inc., Framingham, MA) at IISER, Pune. The instrument was calibrated to less than 10 ppm accuracy by using a calibration mixture of known standard peptides within a mass range of 800 to 4000 m/z. Samples were analyzed in positive ion reflector mode. Data dependent tandem mass spectrometry of 20 most intense peptides from MS scan (PMF) of individual protein was performed with 0.6 μl reconstituted extract mixed with a 0.6 μl fresh α-cyano-4-hydroxycinnamic acid (CHCA) (Applied Biosys-

tems, Framingham, MA) matrix on a 384-well MALDI target plate. PMF and MS/MS data were analyzed with global proteomic solutions software (GPS Explorer, version 3.6; Applied Biosystems, Framingham, MA) and further interrogated for protein identification with the SWISSPROT database by using Mascot search engine. The search parameters for protein identification were set as follows; fixed and variable modifications were carbamidomethyl cysteine and methionine oxidation respectively. Mass tolerances of 50 ppm, requirement of at least five peptides matched, and a maximum of two missed cleavages, were used for protein identification. A protein was considered to be identified correctly with a Mascot score of above 60. Proteins isolated by affinity chromatography were identified by nano-LC-MS^E approach using nano-Acquity-Synapt-HDMS system (Waters Corporation, Milford, MA, USA). Proteins eluted by affinity chromatography were digested in solution. Reduction and alkylation of proteins was carried out by using DTT and iodoacetamide respectively and digested with trypsin. 4 μl of peptides was loaded on the nano-LC. Peptides were trapped on a 5 μM Symmetry C18 column (180 μM 20 mm) and washed for 3 min at 5 μl/min with mobile phase A (0.1 % formic acid). Peptides were then separated and eluted for MS analysis using a 60 min reverse phase gradient at 400 nl/min (5$-$50 % ACN over 35 min) on a BEH 130 C18 1.7 μM x 100 μM x 100 mm nanoAcquity UPLC column. The column temperature was set at 35°C. The reference ([Glu1]-fibrinopeptide B, 500 fmol/ml) was constantly infused by the NanoAcquity auxiliary pump at a constant flow rate of 500 nl/min at an interval of 20 seconds. The eluted peptides spectra were acquired by Synapt-HDMS (Q-TOF) with following parameters. Sample was analyzed in positive V mode in a mass range of 50$-$2000 m/z with a scan time of 1.5 s. The on-line eluted peptides were analyzed at both low collision energy (4 eV) and high collision

energy (15−35 eV). LC-MS^E data were processed with ProteinLynx GlobalServer v2.3 (Waters Corporation, Milford, MA, USA) and searched with *Saccharomyces cerevisiae* data base (UniProtKB) for protein identification [18, 19]

2.2.8 Fluorescence Quenching by Gatifloxacin

To study the interaction of gatifloxacin with its interacting proteins, fluorescence quenching was studied in presence of different concentration of gatifloxacin. A Varian Cary Eclipse fluorescence spectrophotometer equipped with 1.0 cm path length with a Varian multicell peltier temperature controller was used to measure the fluorescence and the fluorescence intensity. Fluorescence quenching spectra were obtained by scanning the emission spectra from 300nm to 500nm at the excitation wave length of 295 nm, with a slit width of 10 nm. The stock solutions of gatifloxacin and its interacting protein were prepared in 50 mM Tris-HCl (pH 7.4)[20].

2.3 Results and Discussion

In this study we have specifically attempted to understand the influence of gatifloxacin on the regulation of proteins involved in glucose metabolism using yeast *Saccharomyces cerevisiae*, which has been considered as a model organism to study glucose metabolism[21, 22]. In order to study the influence of gatifloxacin on proteins involved in glucose metabolism MIC_{50} of gatifloxacin was determined by its ability to inhibit yeast growth rate by 50% after 18 h [17]. The MIC_{50} of gatifloxacin was found to be 154 mg/L (Fig 2.3); the same concentration was used to study the effect of gatifloxacin on glucose utilization and differential protein expression. Glucose

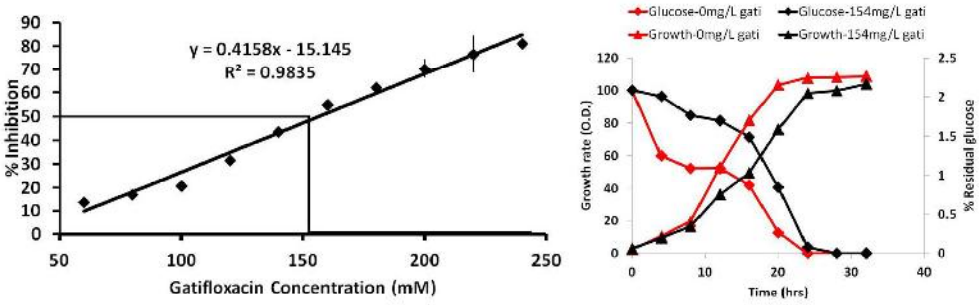

Figure 2.3: **MIC of gatifloxacin (left) and Percent residual glucose in the YPD medium of yeast cells grown in presence or absence of gatifloxacin(right),** Black color represents for 154 mg/L gatifloxacin and red color represents 0mg/L gatifloxacin. Growth curve depicted on secondary y axis and glucose utilization on primary y axis. (n=3)

utilization by yeast was monitored by residual glucose in the medium. In presence of gatifloxacin per cent residual glucose was higher in the medium compared to control at similar number of yeast cells. For example control yeast cells showing absorbance of 1.0 had lower residual glucose than that of yeast cells grown in presence of gatifloxacin, suggesting that the glucose utilization was reduced in presence of drug (Fig 2.3). Further, gatifloxacin induced differentially protein expression was studied using two dimensional electrophoresis and mass spectrometry. Differentially expressed proteins were listed in Table 2.1, and those specific to glucose metabolism were depicted in Fig 2.4. Many of the differentially expressed proteins were involved in glucose metabolism; few proteins were involved in stress response, and gene regulation. Nine proteins identified were involved in glucose metabolism especially six of them belong to glycolytic pathway including alcohol dehydrogenase, triose phosphate isomerise, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), enolase 1, enolase 2, fructose bisphosphate aldolase, pyruvate decarboxylase isozyme 1 (PDC Isozyme 1). Expression of phosphofructok-

Table 2.1: **Gatifloxacin induced differentially expressed proteins in *S. cerevisiae*.** The protein fold change above 1.5 and below .75 where listed with a p value (student's t-test). (n=3) UniProtKB accession numbers were given along with its coverage and probability

Sl. No.	Protein ID	Protein	Protein Score	Protein Score C.I.%	Biological function	Fold change p <0.05
1	P10591	Heat shock protein SSA1	145	100	Stress response	Absent
2	P34760	Thiol-specific antioxidant protein	90	100	Stress response	Absent
3	P16862	6-phosphofructokinase β subunit	135	100	Glucose metabolism	0.28 $\pm$0.12
4	P00942	Triosephosphate isomerase	167	100	Glucose metabolism	0.38 $\pm$ 0.16
5	P00359	GAPDH 3	154	100	Glucose metabolism	0.45 $\pm$ 0.05
6	P20081	FK506-binding protein 1	73	99.974	Glucose metabolism	0.52 $\pm$0. 1
7	P00925	Enolase 2	116	100	Glucose metabolism	0.625 $\pm$0.08
8	P00924	Enolase 1	227	100	Glucose metabolism	0.667 $\pm$0.17
9	P06169	PDC isozyme 1	194	100	Glucose metabolism	2.01 $\pm$0. 21
10	P00330	Alcohol dehydrogenase I	64	99.812	Glucose metabolism	1.99 $\pm$0.19
11	P32324	Elongation factor 2	82	99.997	Protein synthesis	1.89 $\pm$0.13
12	P14540	Fructose-bisphosphate aldolase	69	99.933	Glucose metabolism	1.66 $\pm$0.19

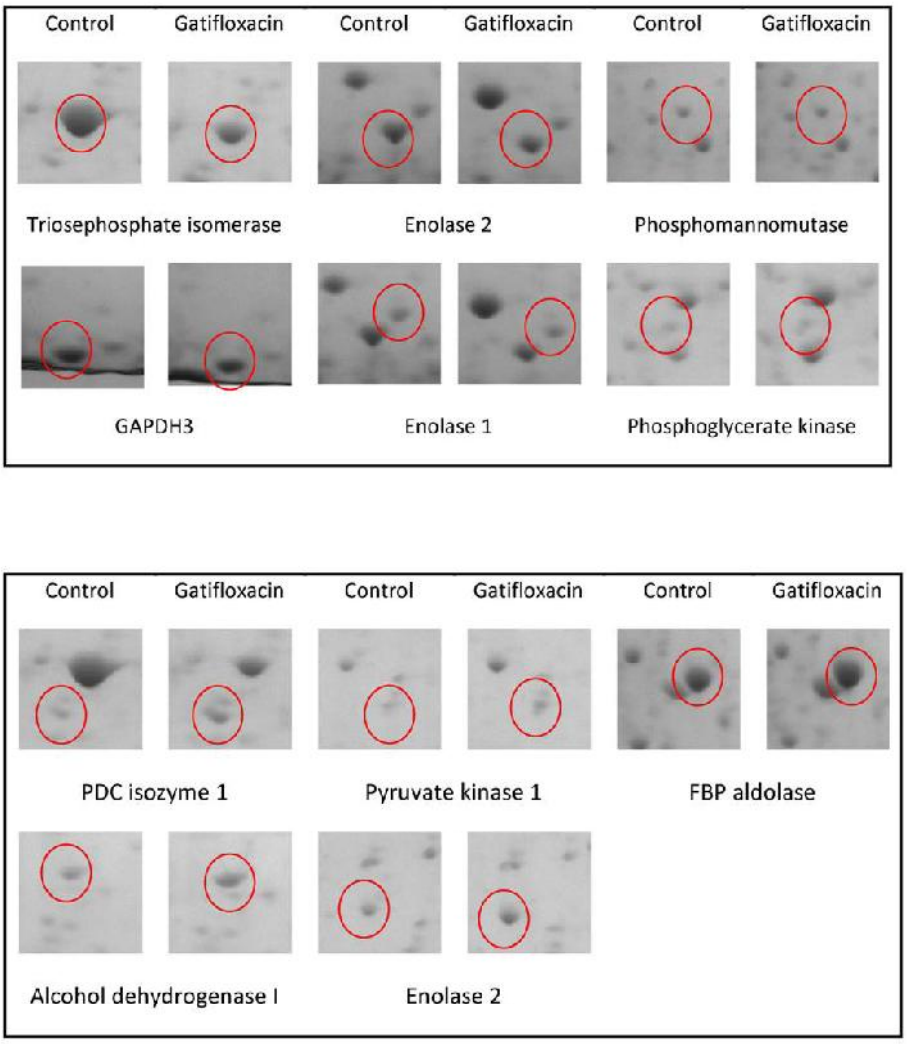

Figure 2.4: **Gatifloxacin induced differential expression of various proteins involved in glucose metabolism in *S. cerevisiae*.** Upper panel represents the down regulated proteins and lower panel represents the up regulated proteins in the presence of gatifloxacin. (n=3)

inase, GAPDH, triose phosphate isomerase, enolase 1 and enolase 2 were down regulated in presence of gatifloxacin. A Similar results were observed in a comparative proteomics study of Pasteurella multocida- a gram negative bacteria, where enorofloxacin, a fluoroquinolone significantly decreased the expression of phosphoenolpyruvate carboxykinase, phosphoglycerate kinase, fructose bisphosphate aldolase, and glyceraldehyde-3-phosphate dehydrogenase [23]. On the other hand in this study, pyruvate decarboxylase isozyme 1, fructose biphosphate aldolase and alcohol dehydrogenase were upregulated by gatifloxacin. By and large, majority of the proteins involved in glucose metabolism were affected by gatifloxacin. Gatifloxacin induced chronic dysglycemic effect in human beings could be due to differential regulation of the enzymes involved in glucose metabolism. Besides proteins involved in glucose metabolism, stress induced proteins such as heat shock protein SSA 1 and thiol specific antioxidant protein were down regulated and were not detected by coomassie staining. The other protein upregulated in presence of gatifloxacin was Elongation factor 2 (EF-2), involved in protein synthesis. In short, the major outcome of the gatifloxacin induced differential expression analysis of protein suggests that the enzymes involved in glucose metabolism are the major targets of gatifloxacin. Chemical proteomics is the method of choice to identify the drug interacting proteins as well as to understand the mechanism of differential regulation of enzymes [24, 25]. In this study we used chemical proteomics approach to understand the molecular mechanism of differential regulation of enzymes involved in glucose metabolism, as well as to identify gatifloxacin interacting proteins. The carboxyl group of gatifloxacin was immobilized onto Affigel-102 (Bio-rad) and its binding proteins were isolated by affinity chromatography Fig 2.2.

By chemical proteomics approach, it was possible to identify Enolase 1

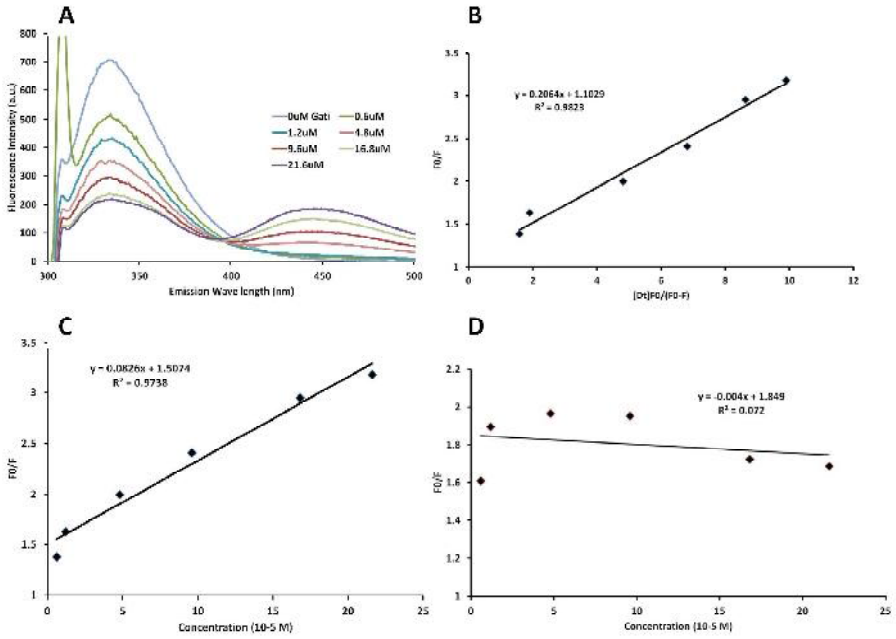

Figure 2.5: **Gatifloxacin Enolase Interaction** A)Enolase fluorescence quenching by increasing concentration of gatifloxacin were 0, 0.6, 1.2, 4.8, 9.6, 16.8, 21.6 μM respectively, B) Scatchard plot depicting binding constant and number of binding sites of gatifloxacin for enolase C)Stern-Volmer Plot depicting dynamic quenching by gatifloxacin D) Stern-Volmer Plot depicting fluorescence quenching by gatifloxacin for Lysozyme.

Table 2.2: **Gatifloxacin binding proteins identified by nano-LC MS^E experiment,** Chemical proteomic approach was used (n=2). The interacting proteins were pulldown by using chemical proteomics approach. Elution of protein was followed by in-solution trypsin digestion and LC-MS^E analysis for protein identification. The identified proteins were represented with UniProtKB accession number.

S. No.	Protein ID	Protein	PLGS Score	Peptides	Theoretical Peptides	Coverage (%)
1	P00924	Enolase 1	320.3118	10	28	42.7918
2	P06169	PDC isozyme 1	178.1635	5	33	19.8934
3	P00360	GAPDH 1	67.7937	4	30	19.8795

(ENO1), glyceraldehyde 3 phosphate dehydrogenase 1 GAPDH), pyruvate decarboxylase isozyme 1 (PDC1) as gatifloxacin interacting proteins (Table 2.2). Enolase was one of the most abundant proteins in the eluate as reflected by more number of peptides and sequence coverage in the LC-MS^E analysis. Therefore, enolase was selected for further *in vitro* binding kinetic studies with gatifloxacin by fluorescence quenching approach. An obvious decrease in tryptophan fluorescence of enolase was observed with increasing concentration of gatifloxacin as depicted in Fig 2.5. However, this trend was not observed with lysozyme, which was used as a negative control for gatifloxacin binding (Fig 2.5). In order to understand the fluorescence quenching mechanism Stern-Volmer equation was plotted as shown in the Fig 2.5 using the following formula 2.1 [20, 26].

$$\frac{F_0}{F} = 1 + K_{SV}[Q] \tag{2.1}$$

Where F_0 and F are the fluorescence intensities in the absence and presence of a quencher (gatifloxacin), K_{sv} and [Q] are the dynamic quenching constant, and concentration of the quencher, respectively. Stern-Volmer con-

stant for enolase upon gatifloxacin binding was 0.82 10-4L.mol-1. Dynamic quenching of fluorescence has been observed in a non-covalent interaction between limofloxacin, an analog of gatifloxacin, and albumin. Therefore, a non-covalent model of Scatchard analysis was used in this study to determine the number of binding sites and binding constant by the following equation 2.2.

$$\frac{F_0}{F} = \frac{K[Dt]F_0}{F_0 - F} - nK[Pt] \tag{2.2}$$

Where, [Dt] is the total drug concentration, [Pt] is the total protein concentration, K is the binding constant and n is the number of binding sites. This equation was graphically represented by plot of F_0/F Vs. $[Dt]F_0/(F_0\text{-}F)$ as depicted in Fig 2.5. The number of gatifloxacin binding sites on enolase is calculated to be 1.102 and the binding constant as 0.261 105L.mol-1. It is evident that the gatifloxacin interacts with enolase by affinity chromatography and fluorescence experiments. However, it is important to establish the biological significance of this interaction. The role of these gatifloxacin binding proteins and probable mechanism of gatifloxacin action has been depicted in Fig 2.6. All the three proteins viz. enolase, GAPDH, PDC isozyme, have regulatory functions on glucose metabolism at different levels including transcription, translation and post translational mechanisms. GAPDH is multifunctional protein, involved in, transcriptional regulation, cell death, cell signalling pathways and glucose transportation [27]. GAPDH may have a role in gatifloxacin induced dysglycemia due to its involvement in insulin signalling pathway, as well as by enhanced glucose transport by influencing GLUT4 activity [28, 29]. Pyruvate decarboxylase isozyme 1 (PDC1) is the other enzyme identified to be gatifloxacin binding protein. It is the key enzyme involved in alcoholic fermentation through the degradation of pyruvate into acetaldehyde and carbon dioxide. Recently, it was shown that the gatifloxacin affects gluconeogenesis by decreasing pyruvate transport to

mitochondria [9]. However, enolase may have a greater role in gatifloxacin induced dysglycemic effect as shown by our study with affinity chromatography and fluorescence quenching, as well as several previous studies indicate a role for enolase in regulation of glucose metabolism. Enolase is involved in several functions ranging from glucose metabolism, transcription to apoptosis [30, 31]. Enolase activity with respect to regulation of glucose metabolism is through mRNA degradation of glucose transporter 1(GLUT1), as well as down regulation of transcription factor c-myc. The mRNA degradation of GLUT1 is carried out by a multienzyme complex called RNA degradosome [30]. Enolase acts as switch to GLUT1 mRNA degradation by binding to RNA degradosome complex. Thus it plays a crucial role in the regulation of GLUT1 mRNA stability. This was evidenced by a recent study, where it was also demonstrated that the gatifloxacin decreases the mRNA levels of the GLUT1 gene [11]. However, the molecular mechanism of gatifloxacin induced GLUT1 mRNA degradation was not been established so far. Gatifloxacin may affect the stability of the GLUT1 mRNA through interaction with enolase, a component of RNA degradosome complex. Additionally, enolase has been shown to down regulate c-myc and thereby decrease the expression of glucose transporter GLUT1, phosphoglucose isomerase, phosphofructokinase (PFK), glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, and enolase. In this study also, it was observed that the expression of ENO, GAPDH, and PFK was down regulated. Enolase controls GLUT1 expression at both transcriptional and translational levels. Together comparative and chemical proteomic approaches suggest that gatifloxacin regulates glucose metabolism at various levels, in addition to its previous role in insulin secretion [7, 8].

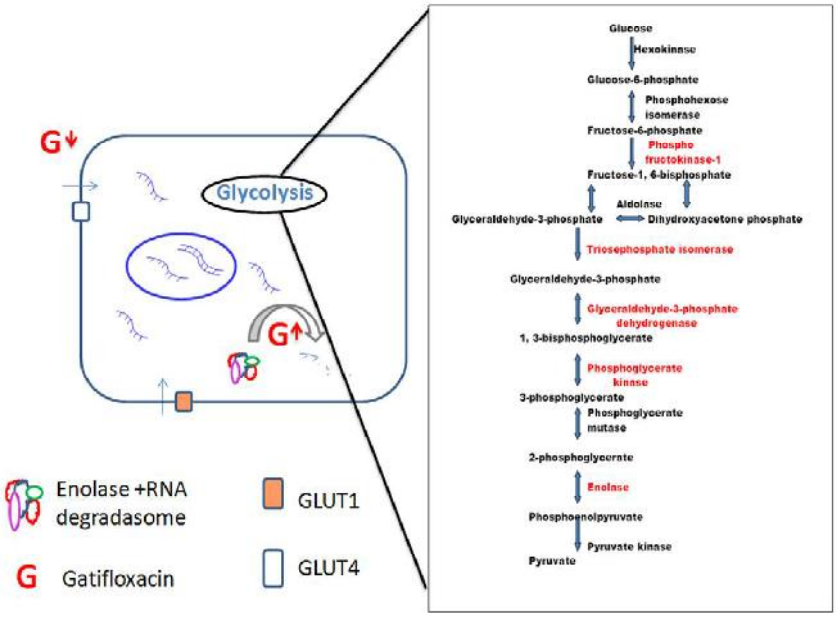

Figure 2.6: **Possible mechanism of gatifloxacin regulation of glucose metabolism.** Gatifloxacin can affect glucose metabolism by three different pathways. They are,(a) Binding of gatifloxacin to enolase, a component RNA degradosome triggers specific degradation of glucose transporter GLUT1. (b) Alternatively gatifloxacin can inhibit GLUT4 *via* insulin dependent pathways, (c) by deregulating, enzymes involved in glycolysis.

2.4 Conclusion

In conclusion, two dimensional electrophoretic studies combined with mass spectrometric analysis suggest that the enzymes involved in glucose metabolism were deregulated by gatifloxacin. Additionally, affinity chromatography revealed that gatifloxacin interacts with enolase, GAPDH, and PDC. Fluorescence quenching experiment further confirms that gatifloxacin interacts with enolase. Both studies, comparative and chemical proteomic approaches suggest that gatifloxacin affects the enzymes involved in glucose metabolism perhaps by regulating at different levels including mRNA degradation, gene expression, glucose transport and metabolism.

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Chapter 3

Proteome Wide Reduction in AGE Modification in Streptozotocin Induced Diabetic Mice by Hydralazine Mediated Transglycation

3.1 Introduction

Glycation is a proteome wide phenomenon resulting from a series of chemical reactions between proteins and reducing sugars leading to formation of heterogeneous Advanced Glycation End products (AGEs). The level of AGEs increases profoundly in diabetes due to chronic hyperglycemic condition. AGEs interact with Receptor for AGEs (RAGE) leading to oxidative stress and activation of pro-inflammatory pathways, which is believed to be the major cause of glycation associated diseases such as diabetic complications, aging,

obesity, inflammation, polycystic ovarian syndrome, ischemic cardiovascular disease, neurodegenerative disorders and cancer. Reducing AGE levels has been considered as an intervention strategy for the treatment of glycation associated diseases [1]. Some of the molecules that reduce AGE levels include aminoguanidine [2], OPB-9195 [3], ALT-946 [4], however, these molecules have not been approved by FDA due to toxic side effects. On the other hand several FDA approved drugs like metformin [5], aspirin [6], diclofenac [7] show anti-glycation activity. All these drugs mainly inhibit the AGE formation; however efforts towards reversing AGE formation are minimal. Interestingly cells have evolved an enzymatic reaction known as deglycation mediated by fructosamine-3-kinase (FN3K)[8, 9] through which AGE formation can be reversed. Deglycation can be achieved chemically by transglycation, where in sugar moiety of Schiff's base/Amadori product is transferred to nucleophiles like free amino acids, polyamines [9]. Glutathione mediated transglycation has been demonstrated *in vivo* [9]. Therefore, deglycation and transglycation are important protective mechanisms against glycation. Development of transglycating agents for preventing or reversing glycation has been hindered due to lack of high throughput assays. Currently available assays for identification of transglycating compounds are based on NMR analysis, which limits a high throughput screening [9]. In this study we have developed mass spectrometry based transglycation assay, using this assay transglycation activity of hydralazine was discovered. Furthermore, hydralazine mediated transglycation was demonstrated in streptozotocin (STZ) induced diabetic mice as evidenced by decreased HbA1c, plasma protein glycation, and formation of hydralazine glucose conjugates in urine. Consequently decreased expression of Receptor for Advanced Glycation End products (RAGE), NADPH oxidase (NOX), and Super oxide dismutase (SOD) was observed with hy-

dralazine treatment. These findings suggested transglycation can be used as an intervention strategy for the treatment of glycation associated diseases such as diabetic complications, atherosclerosis, and aging.

3.2 Materials and Methods

3.2.1 Materials

All chemicals were procured from Sigma-Aldrich unless otherwise mentioned. Hydralazine was generous gift from Dr. M. K. Gurjar, Director (R&D), Emcure, Pharmaceuticals, and Pune. Antibodies for CML, RAGE, NOX, and SOD were procured from Abcam (UK), pentosidine antibody from Cosmo Bio (Japan) and AGE antibody, secondary antibody-biotin conjugate, secondary antibody-HRP were purchased from Merck Millipore, (India), GOD-POD assay kit purchased from Beacon Diagnostics Pvt. Ltd, (India).

3.2.2 MALDI-ToF-MS Based Insulin Transglycation Assay

Insulin was glycated as described earlier [10]. The glycation of insulin was monitored for 710 days till the relative intensity showed 50% on MALDI-TOF-MS (Voyager, ABSCIEX). The excess glucose was removed by dialysis against MilliQ water, lyophilized and stored at -80°C until use. Removal of glucose was confirmed by GOD-POD assay (Beacon). The transglycation assay was performed by incubating 50 μl of 1 mg/ml glycated insulin with 50 μl of 25 mM and 50 mM of hydralazine at 37°C for three hours and analyzed by MALDI-TOF-MS.

3.2.3 Glycation of Human Serum Albumin (HSA)

In vitro glycation of pure HSA was done as described [11] with slight modifications. Briefly, The reaction was carried out by incubating 200 μl of 50 mg/ml HSA in 0.1 M phosphate buffer (pH 7.4) and 100 μl 0.5 M D-glucose containing 5 mM sodium azide as a bacteriostat with or without 100 μl of 25 mM and 50 mM (final concentration) of hydralazine at 37°C for 30 days. HSA glycation was monitored by fluorescence spectroscopy [12] and LC-MS^E [13, 14].

3.2.4 Animal Experiments and Sample Preparation

The animal experiments were approved by Institutional Animal Ethics committee of National Centre for Cell Sciences, India. The experimental protocols were carried out in accordance with the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), India.

Male BALB/c mice were injected with 50 mg/kg body weight of streptozotocin (STZ) in 50 mM citrate buffer pH 4.5 for five consecutive days to induce hyperglycemia, whereas control mice were injected with 50 mM citrate buffer [15]. The induction of diabetes was confirmed after 30 days by measuring the blood glucose levels with Glucometer (Bayer, Germany) and HbA1C level by using HbA1c kit (Bayer Germany). Animals having blood glucose level above 6.12 $\pm$ 1.7 mmol/L with $\geq$7.0 HbA1C were selected for further studies. For each treatment eight animals were grouped into control, diabetic and diabetic treated with either hydralazine (300 mg/L)[26] or aminoguanidine (2.4 g/L)[16] for 60 days after induction of diabetes. Hydralazine and aminoguanidine was made available through drinking water as

described [16, 17]. Glucose and HbA1c levels were monitored on 30^{th}, 45^{th}, 60^{th} and 90^{th} day (0^{th}, 15^{th}, 30^{th} and 60^{th} day after initiation of drug treatment, respectively). Animals were euthanized at the end of study after 60 days of drug administration. Blood samples were collected and were immediately analyzed for blood glucose and HbA1c. Urine was collected in the last week of the experiment by maintaining them in Nalgene metabolomic cages after acclimatization for 24 h. Collected urine was stored at -80°C till analysis [17]. Plasma was obtained by EDTA treatment, which was then centrifuged at 1500 g for 5 min, and the supernatant was stored at -80°C until further use. Total cholesterol, triglycerides, creatinine, and blood urea nitrogen (BUN) concentrations were determined in plasma, and protein and creatinine concentrations in urine with an automatic analyzer (Synchron CX7, Beckman Coulter Inc., Fullerton, CA). Kidney tissue was collected by snap freezing into liquid nitrogen. Later the tissue was perfused with cold phosphate buffer saline to remove blood stains prior to homogenization. The tissue was homogenized to fine powder in liquid nitrogen and the protein was extracted in buffer consisting of 7 M urea, 2 M thiourea, 2% CHAPS, 1% DTT, 40 mM Tris, and centrifuged at 14,000 g for 30 min at 4°C. The supernatant was collected and stored in aliquots at -80°C. Protein concentration was determined by using Bio-Rad protein assay kit (Bio-Rad, Hercules, CA).

3.2.5 NBT Colorimetric Fructosamine Assay

Plasma fructosamine level was measured by using the NBT assay as described previously [18]. 200 μl of 0.75 mM NBT was added to a 96-well microplate containing 10 μl of the plasma. The reduction of NBT by fructosamine group was measured at 540 nm after incubation at 37°C for 30 min using an iMark

microplate absorbance reader (Bio-Rad, Hercules, CA).

3.2.6 LC-MS^E, Protein Identification, Database Search, and PTM Analysis

Prior to LC-MS^E analysis plasma proteins and HSA were digested with trypsin after reduction and alkylation with 100 mM DTT and 200 mM iodoacetamide respectively. Two μl (100 ng/μl) of digested peptides was spiked with 100 fM of enolase, was analyzed by using online nanoACQUITY UPLC coupled to a SYNAPT HDMS (Waters Corporation, Milford, MA) by MS^E [13]. After MS^E analysis, data was analyzed with Protein Lynx Global Server software (PLGS version 2.4 Waters Corporation, Milford, MA). A preliminary search of processed samples was performed for protein identification against the UniProt mice database containing 24382 protein entries. Glycation modification sites were identified by subjecting PLGS search against subset of protein databases that were identified in the preliminary search as described [13, 14]. The false positive rate was set to 4% in the PLGS workflow. The PLGS identified glycation modifications were validated by following criteria. (a) accurate shift in precursor ion mass due to glycation (b) MS/MS spectra of glycated peptides should have at least seven fragments (c) the MS/MS spectra was analyzed for fragment mass error and fragment retention time error (d) glycated peptides should exist in at least two replications. An example of annotation of glycation modification has been shown in the figure 3.1.

Table 3.1: **AGE modifications and their corresponding ΔM**

Sl.no.	Modification	Amino acid	Δ M in Dalton
1	Amadori	K or R	162.0211
2	Carboxy Methyl Lysine (CML)	K	58.0055
3	Carboxy Ethyl Lysine (CEL)	K	72.0211
4	Pyraline	K	108.0211
5	AFGP	K or R	270.074
6	Imidazolone-B	R	142.02
7	MOLD	K or R	49.0078
8	Crossline	K or R	252.11
9	Fructoselysine-1H_2O	K	144.04
10	Fructoselysine-2H_2O	K	126.03
11	MODIC	K or R	36.02

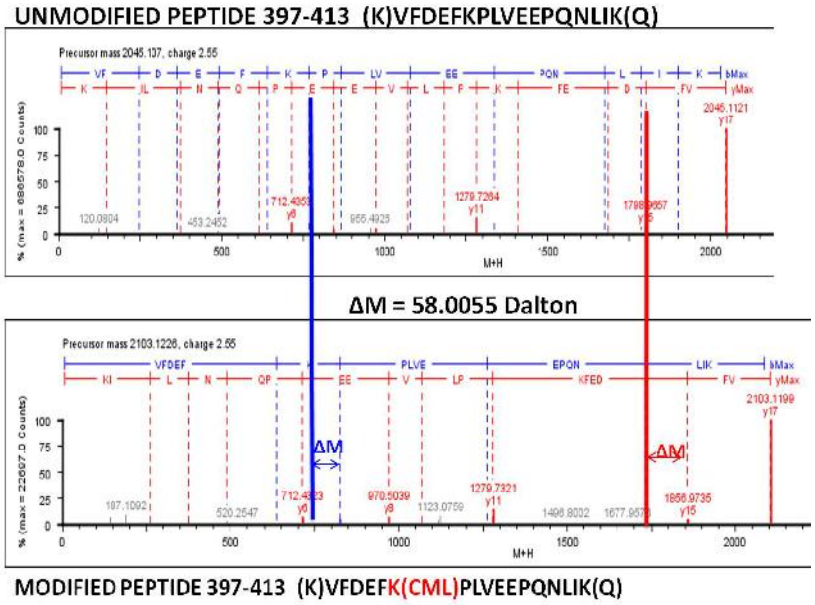

Figure 3.1: **Representative MS/MS spectra of modified and normal peptide.**LC-MS^E spectra of CML modified peptide identified from Human Serum Albumin(amino acid residues 397-413;precursor m/z - unmodified peptide is 2045.107 and CML modified peptide is 2103.1255). Mass increment for CML modification is 58.0055 Da at the amino acid residue of K (402).

Table 3.2: **Glucose Sensitive Amino acid Residues (GSARs) in Human Serum Albumin and their homologous in Mouse Serum Albumin.**

Sl.No.	Sequence	Amino acid location in Human Serum Albumin	Amino acid location in Mouse Serum Albumin
1	HFK	K44	K44
2	VAR	R168	R168
3	VKE	K210	K210
4	LTK	K264	K264
5	KAP	K438	K438
6	AAR	R452	R452

3.2.7 Extent of AGE Modification of Serum Albumin

The extent of glycation modification was determined by analyzing the AGE modification of glucose sensitive peptides of serum albumin as described [19] with a slight modification in table 3.2. We have monitored the AGE modification of all the peptides containing the Glucose Sensitive Amino acid Residues (GSARs) (K44, R168, K210, K264, K438, R452) since trypsin digestion can generate different peptides containing these amino acid residues. The Cumulative Intensity Ratio (CIR) of AGE modified peptides containing GSAR to their unmodified form was monitored to quantify the extent of AGE modification. The CIR was used because the same GSAR can undergo various AGE modifications e.g. CEL, CML etc., and therefore it becomes important to consider the intensity of all the AGE modified peptides. The Cumulative Intensity Ratio (CIR) of AGE modified peptides containing GSAR to their unmodified form was monitored to quantify the extent of AGE modification.

3.2.8 Enzyme-linked Immunosorbent Assay

Each well of a 96-well microtiter plate was coated with 0.1 ml of 20 μg/ml of plasma/kidney protein sample in 50 mM carbonate buffer (pH 9.6) and incubated overnight at 4°C. The plate was washed three times with PBS containing 0.05% Tween 20 (Buffer A). Each well was blocked with 0.5% gelatin, washed with Buffer A, and reacted with 0.1 ml of anti-AGE antibody (0.5 pg/ml). Then the wells were washed with buffer A, incubated with secondary antibody conjugated to alkaline phosphatase (Bangalore Genei, India) for 30 min. Thereafter the plate was washed and incubated in dark with PNPP (p-nitro phenyl phosphate) for 15 min, and the reaction was stopped by addition of sulphuric acid (0.5 M, 10 μl per well). The absorbance of each well was recorded at 415 nm with an iMark microplate absorbance reader (Bio-Rad, Hercules, CA).

3.2.9 Western Blot Analysis

Plasma and kidney proteins were separated on 10% and 12% SDS-PAGE respectively and transferred onto the PVDF membrane. The membranes were blocked with 5% skimmed milk powder dissolved in TBS (20 mM Tris-HCl (pH 7.5), 0.15 M NaCl). Plasma proteins were probed for AGE modification by western blot analysis using antibodies against AGE, CML, and pentosidine antibodies. While kidney proteins were probed for AGE modification, RAGE, NOX and SOD. Antibody against actin served as loading control. Antibody dilution was adopted according to the manufacturer instructions. Corresponding biotinylated secondary antibody was used in 1:2000 dilution followed by incubation with streptavidin HRP conjugate Immunoreactive bands were visualized using DAB system (Sigma) and documented by Bio-

Rad G-800 densitometer.

3.2.10 Synthesis of Hydralazine Glucose and Hydralazine ^{13}C-Glucose Conjugates

Hydralazine glucose conjugate (aldehydo-D-glucose phthalazin-1-ylhydrazone) was prepared by incubating 0.51 mmole of D-glucose and hydralazine hydrochloride with sodium acetate (1.02 mmole) in water: methanol (1:3)[20]. The reaction mixture was kept at boiling water bath for 10 min. Further the reaction was continued at room temperature for 16 hours. The product was filtered, washed with methanol and dried to obtain yellow, needle-like crystals of hydralazine glucose conjugate with quantitative yield. The product was characterized by FT-IR (Fourier Transform Infrared spectroscopy) Vmax 3306 (broad, NH and OH) 1647 (C = N) and 1563 cm-1(Ph) and HRMS (high resolution mass spectrometry) with accurate mass of 323.1384. (Figure 3.2). The same reaction conditions were used to synthesize hydralazine-^{13}C-glucose conjugate.

3.2.11 Urine Analysis for The Identification of Hydralazine Glucose Conjugates

To analyze hydralazine glucose conjugate in urine, urinary proteins were removed by ultrafiltration with 3 kD molecular weight cut-off filters. The filtrate was collected and kept at -80°C until use. The urinary metabolites were separated and analyzed as described earlier [**?**]. In brief 2 μl of sample was separated by Accela UPLC (Thermo Scientific) consisting of binary solvent system and a reverse phase Hypersil GOLD C18, 8 μm, 4.6 $\times$ 150 mm. The binary solvent system included 99.9% water and 0.1% formic acid

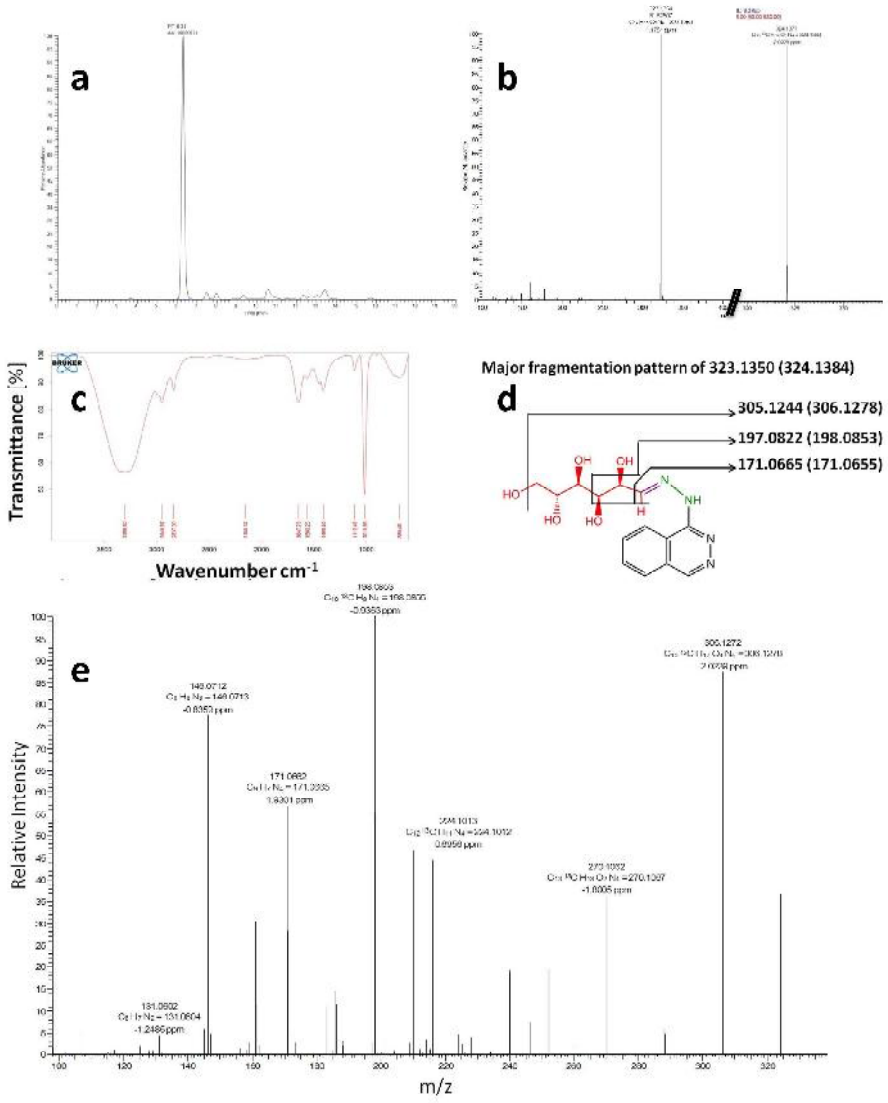

Figure 3.2: **Hydralazine glucose conjugate characterization.** a)LC-MS Chromatogram of HYD-GLU b)Accurate MS measurement of HYD-GLU and HYD-GLU C^13 labeled c) Fourier transform infrared (FT-IR) spectrum of Hydralazine glucose conjugate, on Bruker Optics ALPHA-E spectrometer with a universal Zn-Se ATR (attenuated total reflection) accessory in the 600-4000 cm1 region. IR vmax 3306 (broad, NH and OH) 1647 (C=N) and 1563 cm-1(Ph).d) MS/MS fragmentation pattern of normal and labeled conjugate e) MS/MS spectrum of HYD-GLU conjugate.

(mobile phase A) and 99.9% acetonitrile and 0.1% formic acid (mobile phase B). Hydralazine glucose conjugate was eluted with a flow rate of 0.5 ml/min using sequential gradient of mobile phase B for 1−15% (0−3 min), 15−50% (3−6 min) and finally 50−95% (6−9 min). The UPLC was connected to online high resolution mass spectrometer Q-Exactive (Thermo Scientific), a hybrid quadrupole orbitrap mass analyzer. The mass spectra were acquired in high resolution (30000 FWHM) mode by using Xcalibur and data was processed by Quant software (Thermo Scientific). The method consisted of full scans and targeted MS/MS of selected precursor ion at a defined mass and retention time, with the following instrument parameters; microscans 1, AGC target $2e^5$ and Maximum injection time 120 ms. The retention time was determined by analyzing both labeled (^{13}C) and unlabeled synthetic hydralazine glucose conjugate.

Hydralazine glucose conjugate was analyzed by constructing the XIC of selected fragment ions namely m/z 305.1244 and m/z 306.1278 of unlabeled and labeled (^{13}C) synthetic hydralazine glucose conjugates (m/z 323.1350 and m/z 324.1384) respectively. The standard curve was developed by plotting the $\log_{10}$ value of area under curve (AUC) of the selected fragment (m/z 305.1244) XIC against $\log_{10}$ value of various concentrations of synthetic hydralazine glucose conjugate ranging from 40 fg to 4 ng. All the XIC were extracted with an accuracy of 3 ppm. An optimum concentration of 500 pg/ml of labeled hydralazine conjugate was spiked as an internal standard into the urine samples. Hydralazine glucose conjugate from urine sample was identified and quantified by comparing the XIC of selected fragment ion (m/z 305.1244).

3.2.12 Statistical Analysis

All experiments were performed in triplicates. Statistical analysis was performed by Student's t-test. Data are expressed as means ± SD. A p-value < 0.05 was considered as statistically significant.

3.3 Results and Discussion

In our previous study we have developed a MALDI-TOF-MS based insulin glycation inhibition assay [10], where in insulin (m/z 5808) was glycated with glucose and the intensity of glycated insulin (m/z 5970) was monitored in presence or absence of drugs. This assay was modified to study transglycation by monitoring release of free insulin (m/z 5808) using glycated insulin (m/z 5970) and nucleophilic drugs as reactants. Using this assay, hydralazine, an anti-hypertensive and vasodilating drug, was identified as a transglycating agent (Fig. 3.3). The transglycation of insulin was also evidenced by formation of hydralazine glucose conjugate (m/z 323. 13) in a MALDI-TOF-MS analysis (Fig. 3.3). Further, the effect of hydralazine on protein glycation was studied both *in vitro*, as well as *in vivo* using STZ induced diabetic mice model system.

Glycation leads to protein crosslinking [45] and formation of fluorescent AGEs [12]. Hydralazine inhibited glycation induced *in vitro* HSA protein cross-linking as analyzed by SDS-PAGE analysis (Fig. 3.4). In addition, the drug decreased AGE fluorescence emission at 440 nm suggesting that it inhibits AGE formation. Hydralazine inhibited the AGE formation in a concentration dependent manner and its inhibition was more pronounced compared to aminoguanidine at the same concentration (Fig. 2b). Hydralazine mediated inhibition of *in vitro* HSA glycation was also studied by LC-MS^E

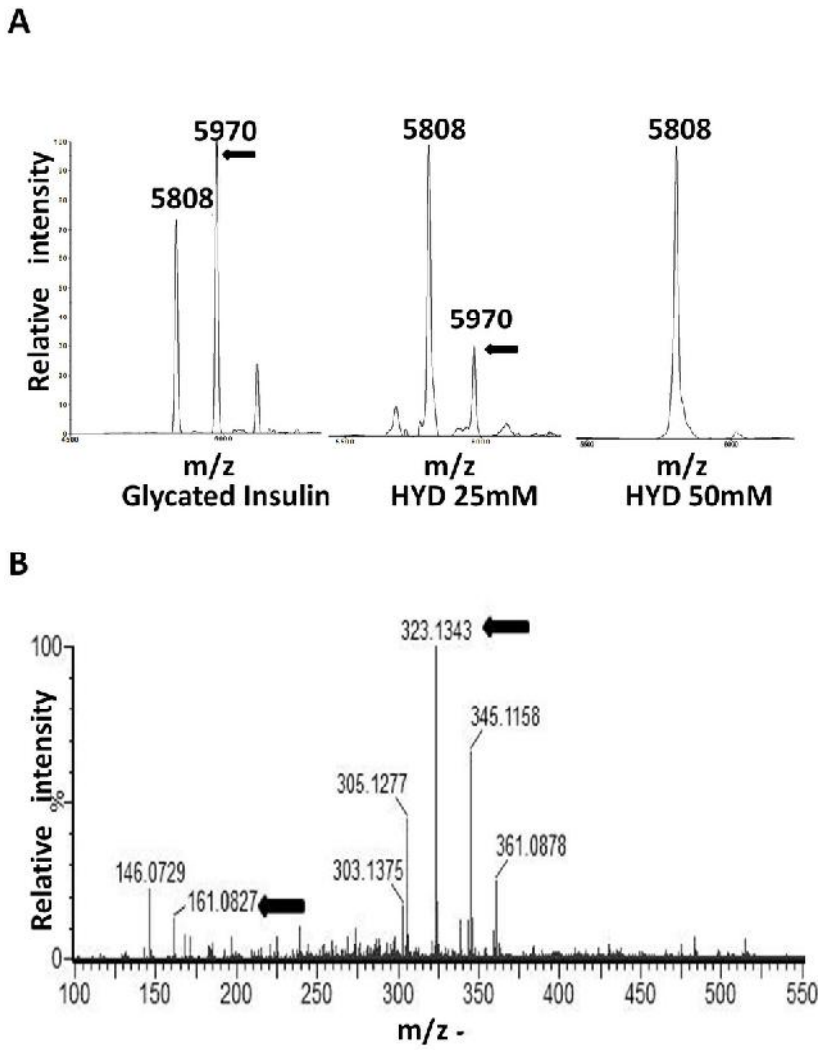

Figure 3.3: ***In vitro* transglycation activity of hydralazine** (a) Glycated insulin (m/z 5970) was incubated with either 0 mM, 25 mM or 50 mM hydralazine for three hours at 37°C and formation of unglycated insulin (m/z 5808) was monitored by MALDI-TOF-MS. Hydralazine showed concentration dependent transglycation of glycated Insulin. (b) MALDI-TOF-MS analysis of hydralazine-glucose conjugate formed during transglycation assay where m/z 161.08 and m/z 323.13Da which are hydralazine and hydralazine-glucose conjugate respectively.

analysis, a data independent acquisition wherein all the eluted peptides are fragmented [21]. This method allowed label free analysis and quantification of even the low intense AGE modified peptides. Previously LC-MS^E has been used to characterize post translational modification namely for demiadation [22], phosphorylation [23], and glycation [13, 14]. Glycated HSA showed more number of AGE modified peptides than normal HSA. The number of AGE modified peptides decreased in presence of hydralazine and aminoguanidine. Hydralazine was more potent than aminoguanidine in inhibition of AGEs as observed by decreased number of AGE modified peptides in LC- MS^E analysis (Fig. 3.4). The representative MS/MS annotated spectra have been shown in Fig. 3.1. Furthermore, the extent of decrease in AGE modification by hydralazine was studied. In a recent study, the extent of glycation of eight glucose sensitive peptides of human serum albumin was monitored for early diagnosis of Type 2 diabetes [19] (Table 3.2). In this study, a similar approach was used albeit with a slight modification, which is described in Material and Methods. As expected the cumulative intensity ratio (CIR) of AGE modified peptides containing Glucose Sensitive Amino acid Residues (GSARs) was highest in glycated HSA than non-glycated HSA. In presence of hydralazine and aminoguanidine, the CIR of GSAR peptides decreased and this decrease was more in hydralazine treatment (Fig. 3.4).

Furthermore, transglycation by hydralazine was demonstrated *in vivo* in STZ induced diabetic mice. Glycation associated parameters such as glycated hemoglobin (HbA1c), fructosamine, plasma AGEs were monitored. STZ induced diabetes led to increase in HbA1c (8.1%), which was decreased significantly in mice treated with hydralazine (Fig. 3.5). The HbA1c decreased with time, and was reversed to near normal levels (4.4%) within 15 days of hydralazine treatment (300 mg/L). However, treatment of aminoguani-

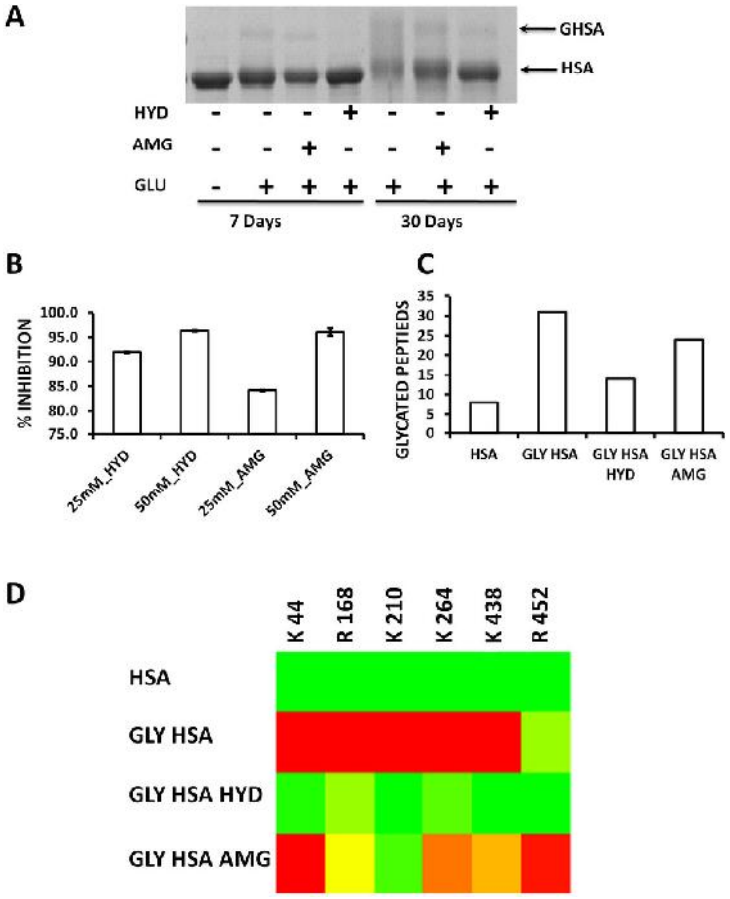

Figure 3.4: ***In vitro* anti-glycation activity of hydralazine.** (a)SDS-PAGE analysis depicting inhibition of glycation induced HSA cross-linking by hydralazine. (b)Hydralazine and aminoguanidine showed glycation inhibition in a concentration dependent manner as observed by decrease in AGE fluorescence emission at 440nm. The bar graph represents the mean values with standard deviation (n=3). (c) LC-MS^E analysis depicting the number of AGE modified peptides in HSA, glycated HSA, glycated HSA in presence of hydralazine (50mM) and aminoguanidine (50mM). In presence of Hydralazine and aminoguanidine the number of AGE modified peptides were decreased. (d) Heatmap analysis of AGE modified GSAR containing peptides. Hydralazine and aminoguanidine treatment decreased the AGE modification of GSAR containing peptides.

dine for 15 days, even at a higher concentration (2.4 g/L) failed to decrease HbA1c (8.4%) (Fig. 3.5). A similar trend was also observed in plasma fructosamine levels (Fig. 3.5). These results suggested that the immediate decrease in glycation can be achieved by transglycation rather than glycation inhibition. Hydralazine also decreased AGE modification of plasma proteins. Serum albumin and few abundant plasma proteins showed enhanced AGE modification in diabetic condition, which were transglycated by hydralazine treatment as reflected by decreased AGE modification in western blotting (Fig. 3.7). The decrease in AGE modification was similar to a relatively higher concentration of aminoguanidine treatment (Fig. 3.7). This trend was also evident in plasma AGE levels quantified by ELISA (Fig. 3.7). Further, LC-MS^E analysis of serum albumin and few abundant plasma proteins revealed relatively lesser number of AGE modified peptides in hydralazine treatment than aminoguanidine treated and diabetic mice (Fig. 3.7 and 3.6).

The extent of *in vivo* AGE modification was studied as described above (Fig. 2d). However, in case of mouse, the GSARs were identified by homology based sequence alignment with human serum albumin (Table 3.2). Heatmap analysis of AGE modified GSAR containing peptides of serum albumin suggested that the extent of modification was highest in diabetes, and was decreased with hydralazine treatment (Fig. 3.6). Accumulation of AGEs in the kidney is associated with development of nephropathic condition [24]. Hydralazine and aminoguanidine decreased AGE levels and AGE modification of kidney proteins in diabetic mice as measured by ELISA and western blot respectively (Fig. 3.8). AGEs are also known to induce increased expression of RAGE, NADPH oxidase and SOD [25]. Hydralazine and aminoguanidine treated diabetic mice kidney showed decreased expression of these proteins (Fig. 3.8). Hydralazine mediated transglycation was also evidenced

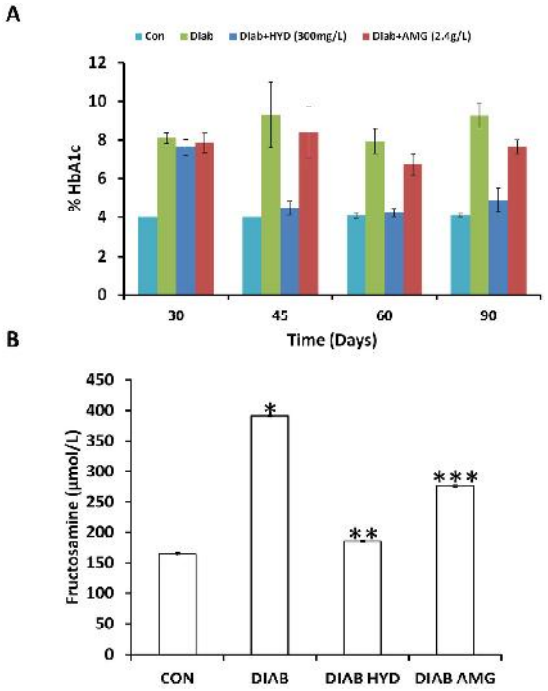

Figure 3.5: ***In vivo* transglycation activity of hydralazine.** (a) Bar graph depicting the HbA1c of control mice, diabetic mice, and diabetic mice treated with hydralazine or aminoguanidine at 30^th, 45^th, 60^th and 90^th day of experiment. Hydralazine decreased the HbA1c by 45^th day (15 days of drug treatment) and was almost equal to that of control mice. The values represent mean±SE, n=6, P- value <0.05. (b) Bar graph depicting the plasma fructosamine levels of control mice, diabetic mice, and diabetic mice treated with hydralazine or aminoguanidine at 90th day of experiment. Fructosamine levels were decreased with hydralazine treatment (The values represent mean±SE, n=6, P- value <0.05).

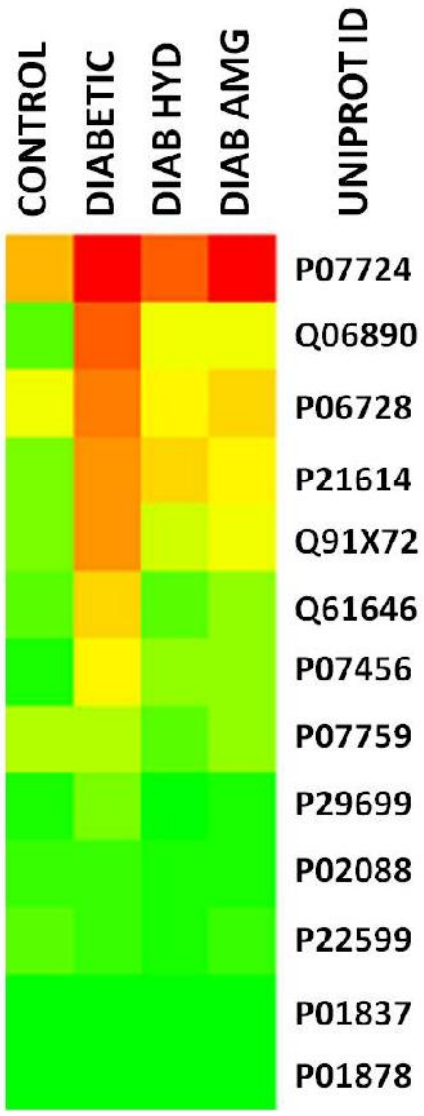

Figure 3.6: **Hheatmap depicting AGE levels of different abundant plasma proteins with respect to diabetes and treatment with HYD and AMG:** Heatmap analysis of PLGS identified AGE modified peptides of abundant plasma proteins, showing differences in their number in control, STZ induced diabetic, Hydralazine (DIABHYD) and Aminoguanidine (DIABAMG) treated diabetic mice. Green color indicates decreased number of glycated peptides, while red colour indicates increased number of glycated peptides. The proteins are represented as UNIPROT accession numbers.

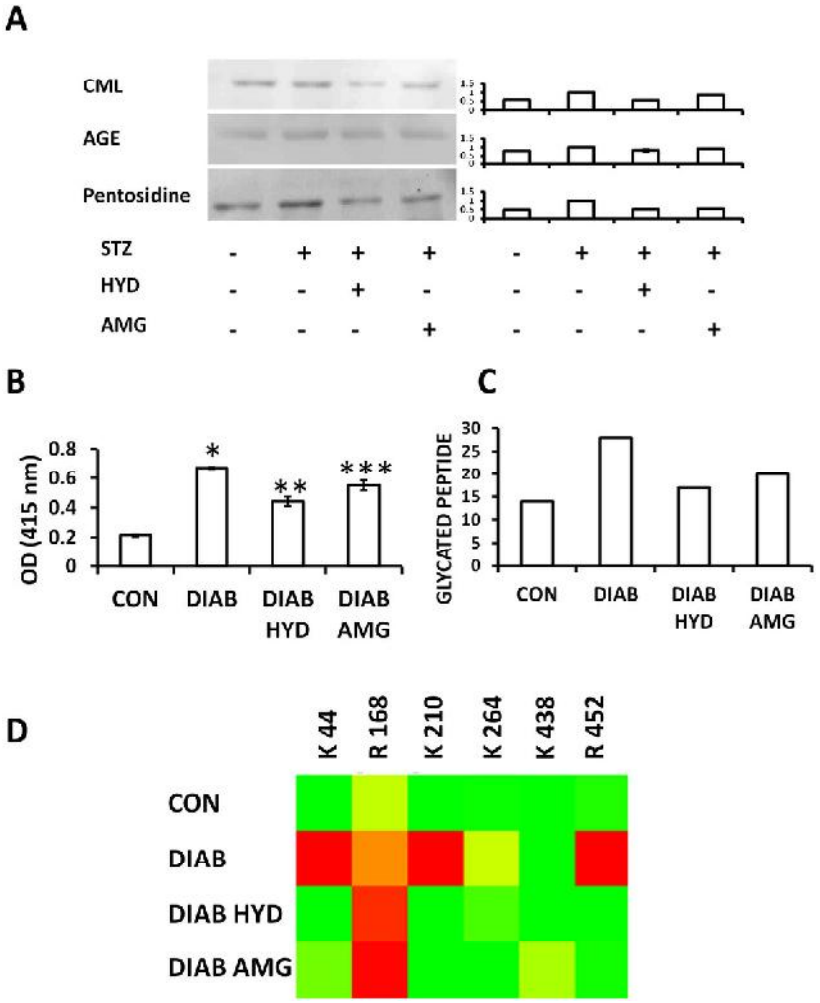

Figure 3.7: **Hydralazine mediated *in vivo* reduction of AGE modification of plasma proteins.** (a) Plasma proteins were probed for AGE modification by western blot analysis using antibodies against CML (top panel), AGE (middle panel), and pentosidine (bottom panel) antibodies. (b) Plasma AGE levels were measured by ELISA. The bar graph depicts decrease in relative AGE levels upon treatment with hydralazine and aminoguanidine. (The values represent mean± SE, n=6, P-value<0.05). (c) Bar graph depicts the decrease in number of AGE modified peptides of serum albumin by LC-MS^E analysis in presence of hydralazine (HYD) and aminoguanidine (AMG) (n=2) (d)) Heatmap analysis of extent of AGE modification of GSAR containing peptides of mouse serum albumin.

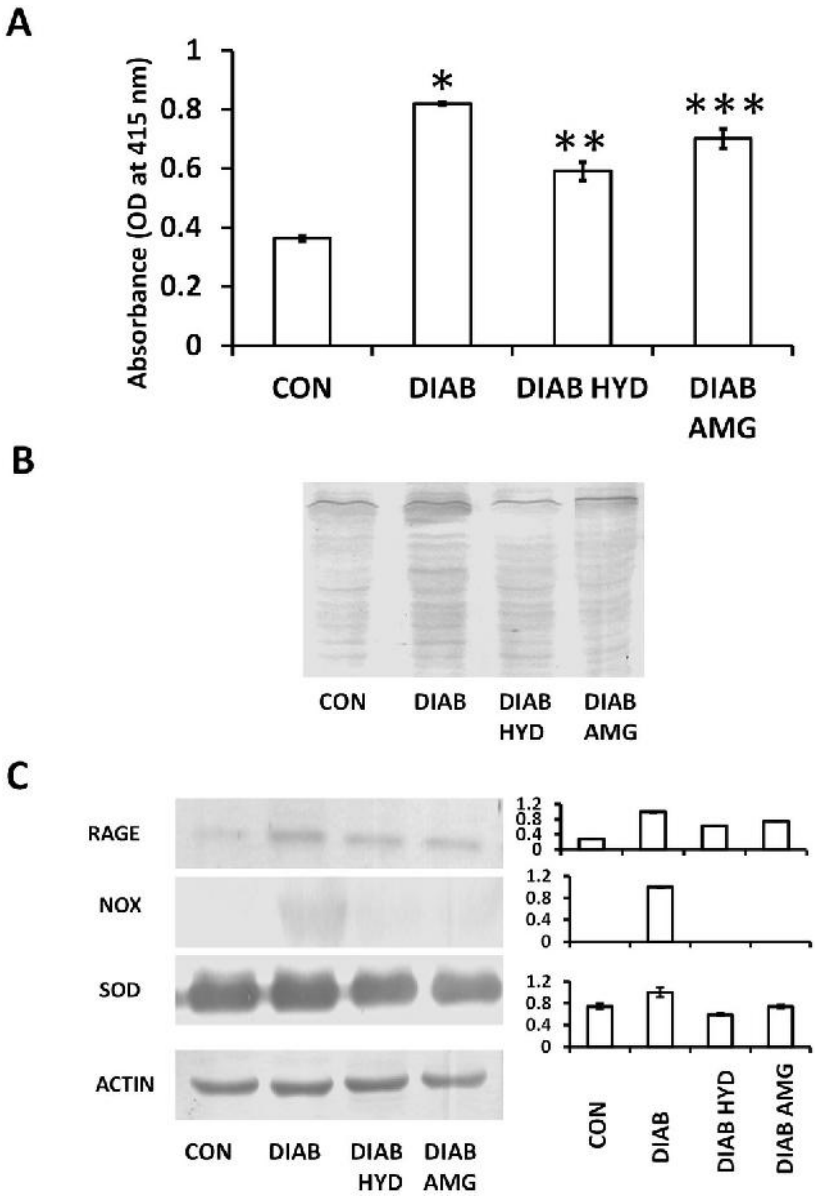

Figure 3.8: **Effect of hydralazine mediated transglycation on kidney proteins.** (a) Kidney AGE levels were measured by ELISA. (The values represent mean± SE, n=6, P- value <0.05). (b) Kidney proteins were probed using AGE antibodies by western blot analysis. Hydralazine decreased the AGE modification of kidney proteins (Lane DIAB-HYD) compared to STZ induced diabetic kidney proteins (Lane DIAB). (c) Western blot analysis of kidney proteins indicating the expression of RAGE, NOX and SOD. Hydralazine and aminoguanidine treatment decreased the expression of these proteins (Lanes DIAB-HYD and DIAB-AMG) as shown by densitometric analysis in the bar graph.

by analysis of hydralazine glucose conjugate in urine using high resolution accurate mass spectrometer. To analyze the hydralazine glucose conjugate, retention time (RT 6.3 min) was determined by spiking its labeled form (hydralazine ^{13}C-glucose conjugate) as an internal standard (Fig. 3.2). The fragmentation pattern of unlabeled and labeled hydralazine glucose conjugate has been shown in (Fig. 3.2. The fragmentation was exactly identical except for few fragments that showed an increase in mass by 1Da e.g. m/z 306.1278 and m/z 198.0853 due to presence of ^{13}C label in those fragments ((Fig. 3.2). A similar fragmentation pattern was observed in urine sample spiked with labeled conjugate (Fig. 3.9). Extracted ion chromatogram (XIC) of selected fragment ion (m/z 305.1244) was used to develop standard curve (Supplementary Fig. 8). Hydralazine glucose conjugate was identified and quantified by monitoring the XIC of its selected fragment ion (m/z 305.1244). About 304.22 $\pm$ 20.35 pg of hydralazine glucose conjugate per milliliter of urine was detected in diabetic mice treated with hydralazine (Fig. 3.9. This drug conjugate was not detected in control mice treated with hydralazine perhaps due to its lower concentration. Hydralazine treatment showed beneficial effects in terms of decreased levels of plasma cholesterol, triglycerides, blood urea nitrogen, and creatinine levels, as compared to diabetic mice and aminoguanidine treated diabetic mice (Table 3.3).

Discussion

In this study we have shown that hydralazine inhibits AGE formation by transglycation. The possible mechanism by which hydralazine causes transglycation is depicted in Fig. 3.10. The reaction between glucose and protein leading formation of Schiff's base and Amadori product is reversible in nature. It is possible that hydralazine interferes at the level of Schiff's base or

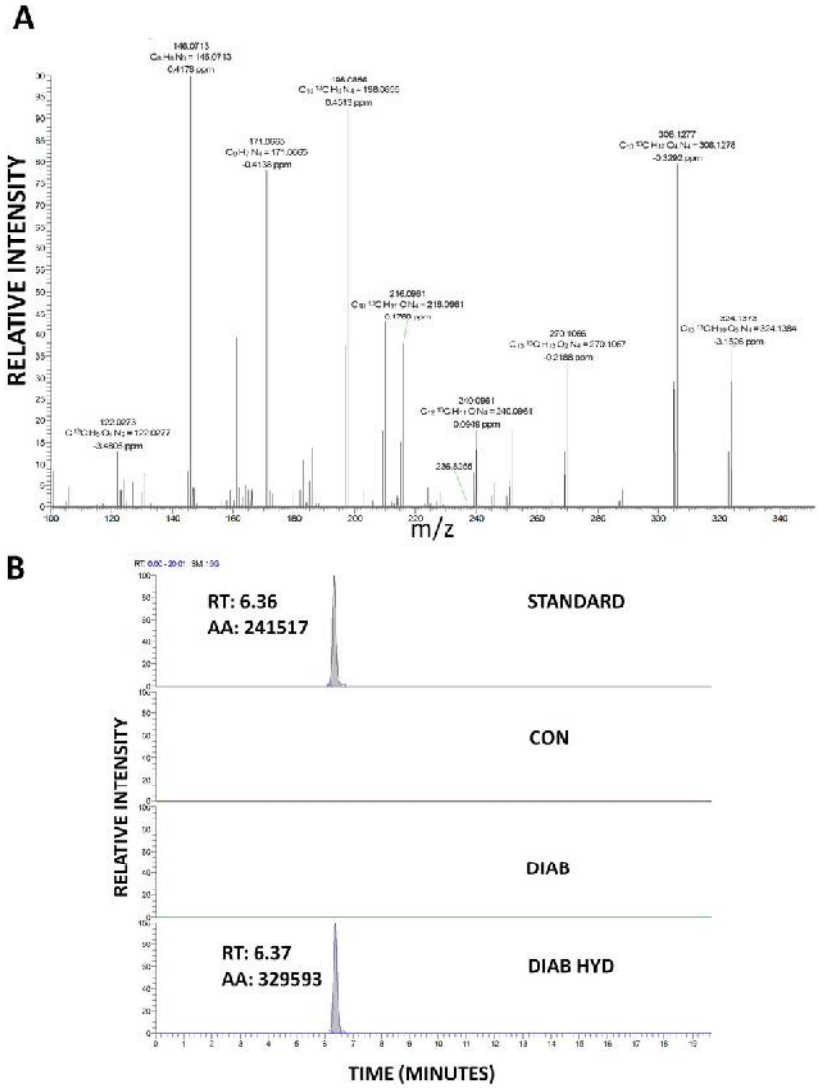

Figure 3.9: **Detection of Hydralazine-Glucose Conjugate in urine of diabetic mice treated with hydralazine** (a) Extracted ion chromatogram (XIC) of hydralazine-glucose conjugate (m/z 323.1335, RT 4.3 min) of synthetic standard, as well as in control, hydralazine treated control, diabetic, and diabetic treated with hydralazine. (b) Mass spectrum of hydralazine glucose conjugate in urine of diabetic mice treated with hydralazine.

Table 3.3: **Blood urea nitrogen (BUN), creatinine, cholesterol and triglyceride levels in plasma of control, diabetic and diabetic treated with hydralazine and aminoguanidine mice.** (The values represent mean±SE, n =6)

Treatment	BUN (mg/dl)	Creatinine (mg/dl)	Cholesterol (mg/dl)	Triglycerides (mg/dl)
Control	23.53 ± 5.64	0.29 ± 0.03	71.00 ± 12.13	107.25 ± 10.01
Diabetes	38.48 ± 15.12	0.37 ± 0.04	118.25 ± 37.05	138.75 ± 21.54
Diabetic+ Hydralaizne	18.33 ± 4.58	0.27 ± 0.02	68.75 ± 14.05	96.75 ± 18.00
Diabetic+ Aminoguanidine	24.47 ± 4.29	0.32 ± 0.05	69.33 ± 14.29	112.00 ± 24.24

Amadori product formation and removes the protein bound glucose by virtue of its nucleophilic nature. A similar mechanism was proposed in case of glutathione mediated transglycation [9]. Previous studies have employed various strategies to inhibit AGE accumulation [27]. These approaches are broadly divided into chemical and cellular approaches. Chemical approaches involve inhibition of Amadori products [28] and AGEs [29], chemical quenching of reactive dicarbonyls [30], cross-link breakers [31]. While, cellular approaches include enzymes such as, aldose reductase [32], which converts reducing sugars to corresponding sugar alcohols; aldehyde dehydrogenases and glyoxalases [33], which convert reactive dicarbonyls to their inactive forms; matrix metalloproteases that are involved in the release of soluble RAGE (sRAGE) from mRAGE, which prevents AGE interaction with mRAGE [34]. Another such enzyme, namely fructosamine-3-kinase is involved in deglycation [9],

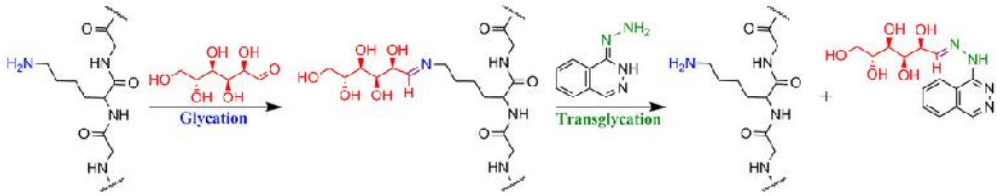

Figure 3.10: **Probable Mechanism of Hydralazine mediated transglycation.** Hydralazine may interfere at the level of Schiff's base or Amadori product formation during glycation reaction and removes the protein bound glucose by virtue of its nucleophilic nature.

which inspired the discovery of small molecule mediated chemical deglycation called transglycation [9]. Biomolecules such as glutathione, cysteine, and polyamines have shown to have transglycation activity [9]. Synthetic molecules such as isoniazid and aminoguanidine show poor transglycation activity [35]. Peptide derivative like N-acetyl carnosine with transglycation activity is being used in the eye drop formulation for the treatment of diabetes induced cataract [36]. Although this is an encouraging indication for use of glycation inhibitors, yet there are no FDA approved drugs. In view of this, discovery of hydralazine as a transglycation agent has a great significance in controlling AGE levels and for the treatment of AGE induced diseases.

Previous studies have shown hydralazine inhibits glycation of low density lipoproteins and prevents foam cell formation in murine macrophages [37]. The drug was shown to inhibit *in vivo* AGE formation, and improved renal damage in a type 2 diabetic nephropathy rat model [38]. These studies supported our finding of hydralazine as a transglycating agent and AGE inhibitor. AGE accumulation is associated with up-regulation of RAGE expression and activation of AGE-RAGE axis leading to oxidative stress. In earlier studies hydralazine treatment resulted in decreased oxidative stress

and lipid peroxidation [39], perhaps this can be attributed to inhibition of AGE formation. Recent studies involving hydralazine as an acrolein scavenger [40] support its ability of transglycation. Therefore, the drug has been suggested for the treatment of atherogenesis [41], nervous system trauma [42] and Alzheimers [43]. In this study for the first time we have unequivocally demonstrated the transglycating ability of hydralazine by *in vitro* and *in vivo* experiments including insulin transglycation by MALDI-TOF-MS, analysis of hydralazine-glucose conjugate, as well as proteome wide decrease in AGE modification of plasma proteins and AGE levels in plasma and kidney. The decrease in AGE modification and AGE levels was quite significant in comparison with a relatively higher dose of aminoguanidine treatment. Furthermore, hydralazine was able to reduce HbA1c and AGE levels quite faster when compared to aminoguanidine suggesting transglycation is a better intervention strategy. As hydralazine is already FDA approved drug for the treatment of hypertension, it has the potential to be repositioned for treatment of glycation induced diseases.

3.4 Conclusion

In conclusion,we are able to show, transglycation activity *in vitro* as well as *in vivo.* Here we report the transglycation activity of hydralazine using a newly developed MALDI-TOF-MS based assay. Hydralazine mediated transglycation of HbA1c, plasma proteins and kidney proteins was demonstrated in streptozotocin induced diabetic mice, as evidenced by decrease in protein glycation, as well as presence of hydralazine-glucose conjugates in urine of diabetic mice treated with hydralazine. Hydralazine down regulated the expression of Receptor for Advanced Glycation End products, NADPH oxidase,

and super oxide dismutase. These findings will provide a new dimension for developing intervention strategies for the treatment of glycation associated diseases such as diabetes complications, atherosclerosis, and aging.

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Chapter 4

Inhibition of Advanced Glycation End Products Formation by Aspirin Mediated Pre-acetylation of Proteins

4.1 Introduction

Glycation is a dynamic chemical reaction between carbonyl group of reducing sugar and amino group of proteins leading to formation of Advanced Glycation End products (AGE). AGEs alter structural and functional activity of proteins [1], interact with RAGE causing oxidative stress [2, 3, 4], and favors cross linking of proteins leading to formation of detergent-insoluble and protease-resistant aggregates [5]. Toxicity of glycated protein may be due to the AGE modification or due to the aggregation. Such AGE modified aggregates are associated with diabetic complications including vascular stiffening [6], atherosclerosis [7], cataracts [8], and a large number of age-

related disorders including Parkinson's disease [9], Alzheimer's disease [10] cancer [11], etc. Glycation induced misfolding and aggregation of proteins is one of the main causes of the age related disorder [12, 13]. Hence, inhibition and modulation of glycation has been proposed as an intervention strategy to treat glycation associated diseases [11]. Glycation can be attenuated by various strategies viz a) Inhibition of Schiff's base formation b) deglycation c) transglycation d) crosslink inhibitors e) Crosslink breakers f) AGE degradation. All these strategies act either at the early stages or advanced stages of glycation reaction, and there are no known mechanisms to prevent/protect proteins from glycation in a glucose living systems. A comprehensive approach which utilizes different mechanisms of inhibition of AGE formation should be used for controlling protein damage. One of the other ways by which AGE formation can be prevented is by blocking the sites of glycation, this can be achieved by post translation modification such as acetylation, hydroxylation, methylation, formylation etc that utilize the sites of glycation and prevent AGE formation. Among them, acetylation can be achieved by simple *in vitro* reaction with acetyl salicylic acid [14] (aspirin), one of most commonly used drug for prevention of cardiovascular diseases. We speculate that aspirin prevents glycation *in vivo*, in addition to its role as platelet aggregation inhibitor [15, 16]. The glycation inhibitory and protective activity of the aspirin has been well studied in the rat lens proteins against different sugars such as galactose [17], glucose [18], glucosamine [19]. *In vivo* studies reported that aspirin fed diabetic rats (200 mg/kg of body wt) showed decrease in HbA1c and total soluble glycated lens proteins compare to the STZ induced diabetic rats [18]. Aspirin treatment protects enzyme like δ-aminolevulinic dehydratase, porphobilinogen deaminase and blocked the accumulation of lipoperoxidative aldehydes which could prevent some of

the late complications of diabetes [20]. In the present study, we tried to understand the effect of aspirin induced pre-acetylation on protein glycation both *in vitro* and *in vivo* by STZ induced diabetic mice model. In our *in vitro* experiments have shown that pre-acetylation of proteins are protected from glycation induced altered secondary structure and function, AGE formation, protein cross-linking and aggregation. Further it is proved by animal experiment also.

4.2 Material and Methods

All chemicals were procured from Sigma unless otherwise stated.

4.2.1 Protein acetylation

50 mM of Aspirin were incubated with insulin (2 mg/ml) in 0.1M phosphate buffer (pH 7.4) for 7 days at 37°C. Same procedure was followed for the HSA (50mg/ml). The acetylated proteins were dialysed and lyophilised and stored at -80°C until used.

4.2.2 Glycation Assay

MALDI-TOF-MS based assay was used to monitor the extent of glycation (Fig. 4.1). Insulin was used as model protein. The final assay concentration is maintained of 0.5M glucose and 1mg/ml of insulin in 0.1M phosphate buffer [21]. The assay was kept for incubation at 37°C for 10 days and analysed by MALDI-ToF (AB SCIEX MALDI-ToF/ToF 5800). The HSA assay was carried out by incubating 200 μl of 50 mg/ml HSA in 0.1 M phosphate buffer (pH 7.4) and 100 μl of 0.5 M D-glucose containing 5 mM sodium azide as a bacteriostat at 37°C for 30 days [22]. Pre-acetylation effects were observed

by using acetylated insulin and acetylated HSA instead off normal insulin or HSA.

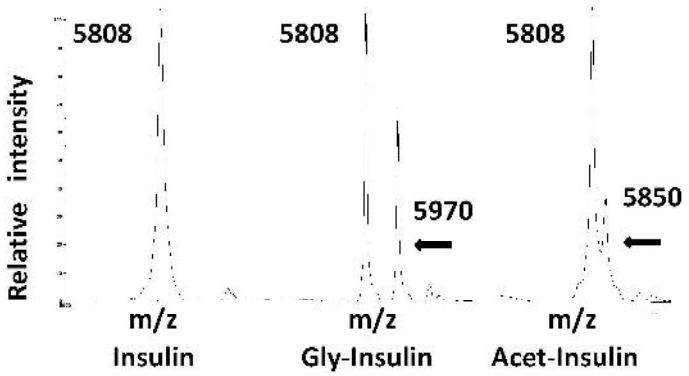

Figure 4.1: ***In vitro* MALDI-MS based protein modification assay.** A) Normal insulin (m/z- 5808 Da), B) Glycated insulin (m/z- 5970 Da) and C) acetylated insulin (m/z- 5850 Da). Glycated and acetylated insulin was formed by incubating with glucose and aspirin, respectively.

4.2.3 Protein Cross-linking and Aggregation

HSA or acetylated HSA was incubated with or without 0.5 M glucose in 0.1M phosphate buffer (pH 7.4) at 37°C for 30 days. Formation of protein cross linking and aggregation was analyzed by CD spectrometry, UV-Vis spectroscopy and SDS-PAGE.The crosslink of protein was monitored by 10% SDS-PAGE. Browning and Carbonylation were measured at 340 nm and 365 nm on UV-1800 spectrophotometer (SHIMADZU).

4.2.4 Circular Dichroism Spectroscopy

HSA was diluted to 200 μml with water, and CD spectra were recorded by using J-720 spectrometer (Jasco) from $200-240$ range. Solutions containing corresponding amounts of Aspirin and ribose in same buffer without HSA

were used as controls. Reference CD spectra were recorded and subtracted from the CD signals to isolate the HSA-specific changes in the CD spectra. The spectral data were submitted to the K2D2 online software to get α-helix and β-sheets composition.

4.2.5 Animal Experiments

The animal experiments were approved by Institutional Animal Ethics committee of National Centre for Cell Sciences, India. The experimental protocols were carried out in accordance with the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), India. Male BALB/c mice were injected with 50 mg/kg body weight of streptozotocin (STZ) in 50 mM citrate buffer pH 4.5 for five consecutive days to induce hyperglycemia, whereas control mice were injected with 50 mM citrate buffer [23]. The induction of diabetes was confirmed after 30 days by measuring the blood glucose levels with glucometer (Bayer, Germany) and HbA1C level by using HbA1C kit (Bayer Germany). For each treatment six animals were grouped into control, diabetic, Aspirin pre-treated diabetic and diabetic treated with Aspirin (200 mg/L) for 30 days after induction of diabetes. Aspirin was made available through drinking water. Glucose and HbA1c levels were monitored on 30^{th}, 45^{th}, and 60^{th} day (0^{th}, $15^{th}$and 30^{th} day after initiation of STZ injection, respectively). Animals were euthanized at the end of study after 30 days of drug administration. Blood samples were collected and were immediately analyzed for blood glucose and HbA1c. Plasma was obtained by EDTA treatment, which was then centrifuged at 1500 g for 5 min, and the supernatant was stored at -80°C until further use. Total cholesterol, triglycerides, creatinine, and blood urea nitrogen (BUN) concentrations were determined in plasma, and (Synchron CX7, Beckman

Coulter Inc., Fullerton, CA). Protein concentration was determined by using Bio-Rad protein assay kit (Bio-Rad, Hercules, CA).

4.2.6 Western Blot Analysis Of Plasma Proteins

Equal amount of plasma protein (10 μg) was separated on 10% SDS- PAGE and then transferred to PVDF membrane (TE 77 Semi- Dry Transfer Unit, GE Healthcare UK Limited), which was incubated overnight at 4°C in blocking buffer containing 5% skimmed milk powder dissolved in PBS. The membranes were washed with PBS and incubated for 1 hrs at 37°C with diluted (1:1000) either anti-CML antibody (Abcam-ab27684) or anti acetyl lysine antibody (Millipore 06−933). After three washes with washing buffer (PBS containing 0.05% Tween-20), the membranes were incubated with goat anti-rabbit IgGs-conjugated to HRP at a dilution of 1:5000 for 60 min at room temperature. Immunodetection was performed by adding Advansta Western Bright kit with substrate of HRP and acquired the images by Syngene Diversity gel documentation system.

4.2.7 Statistical Analysis

All experiments were performed in triplicates. Data are expressed as means ± SD. A p-value < 0.05 was considered as statistically significant.

4.3 Result and Discussion

4.3.1 Protection from Glycation by Pre-acetylation in Insulin and HSA

Protein glycation has been implicated in development of diabetic complications and neurodegenerative diseases, it is important to develop therapeutic strategies for the treatment of these diseases through glycation inhibition. Several known drugs such as metformin [24], diclofenac [25], ibuprofen, aspirin [19] have shown to glycation inhibition activity. Aspirin is being used in the treatment of cardiovascular diseases due to its antithrombotic effects through platelet-independent mechanisms [26]. Previous studies have shown that aspirin has the ability to acetylate proteins [16]. In this study we have addressed whether pre-acetylation of proteins can protect them from glycation reaction. In order to show acetylation protects glycation, insulin and HSA were used as model proteins. *In vitro* protein glycation is analysed by SDS-PAGE, MALDI-ToF MS, carbonylation, browning and protein structure by CD spectrometry. In our previous studies we have used MALDI TOF-MS to study glycation of insulin [21]. The glycation of insulin can be observed as an increment of mass by 162 Da and acetylation can be observed as increase in 42 Da. In the *In vitro* assay, we are able to observe that normal insulin shows higher amount of glycation than pre-acetylated insulin (Fig. 4.2). These results imply that pre-acetylation prevents binding of glucose to the protein and thereby protects from glycation. Similar results were observed with HSA in browning and carbonylation experiments (fig 4.3). Further it is clear from CD analysis and SDS-PAGE.

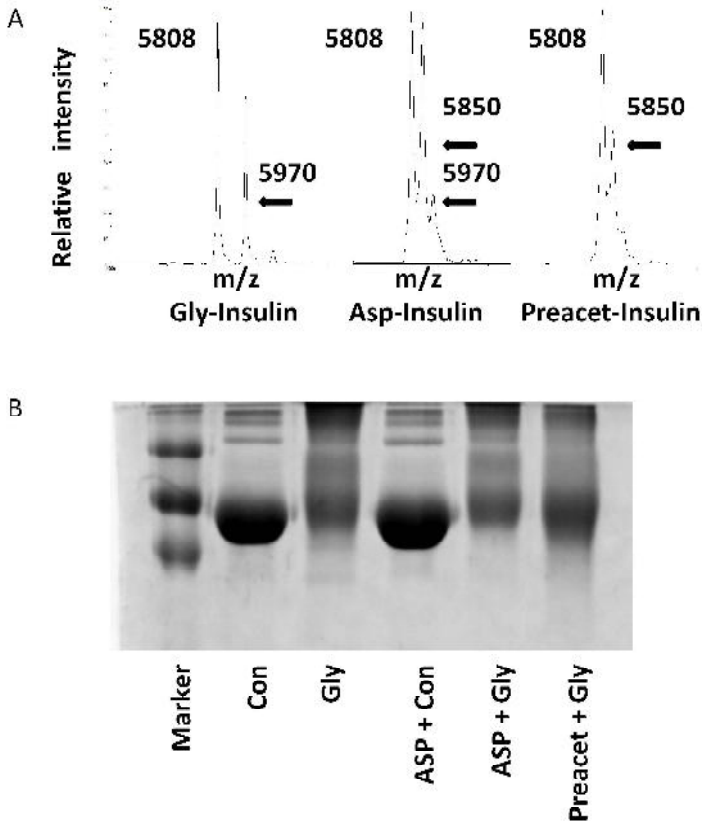

Figure 4.2: ***In vitro* pre-acetylation assay with Insulin and HSA.** A) Insulin glycation assay. Assay is performed with incubating the proteins with 0.5M glucose for 10 days. Glycation is observed as the relative intensity of glycated insulin with m/z- 5970 Da. Pre-acetylated insulin was better inhibited the glycation than glycation inhibition by aspirin B) HSA Glycation Assay by SDS-PAGE Glycation leads to protein cross linking and aggregation. This can be observed as smear formation in SDS-PAGE (lane B). Here we observed that pre-acetylation inhibits cross link formation (lane E) than glycation inhibition by aspirin (lane D).

Table 4.1: Blood urea nitrogen (BUN), creatinine, cholesterol and triglyceride levels in plasma of control, diabetic and diabetic treated with aspirin and pre-acetylated mice. (The values represent mean SE, n = 6)

Treatment	BUN (mg/dl)	Creatinine (mg/dl)	Cholesterol (mg/dl)	Triglycerides (mg/dl)
Control	25.13±1.80	0.25±0.020	78.94±3.60	100.27±9.22
Diab	40.53±3.23	0.37 ±0.027	127.6±7.45	140.81±5.93
Con+Aspirin	25.59±2.76	0.29±0.035	77.00±8.27	95.37±20.19
Diab+Aspirin	31.99±2.15	0.32±0.028	108.86±9.03	115.22±8.54
Preacety+Diab	27.17±2.36	0.25±0.024	84.74±5.85	107.31±7.93

4.3.2 Effect on Secondary Structure of The Protein

Carbonyl group of glucose reacts with an amino group of a protein, and water is eliminated to form fructosamine, which is considered as early product of the glycation [27]. Further glycation leads to formation of AGEs, change in secondary structure of proteins as formation of beta sheet, protein cross-linking, aggregation [1, 28]; therefore we analyzed these properties after pre-acetylation of protein with aspirin. It is reported earlier that reducing sugars like glucose induces the secondary structural changes in the HSA [1]. The CD spectroscopy suggested increased beta sheet formation in glycated proteins was protected by pre-acetylation. Figure 3 depicts the percentage of alpha and beta sheet composition in the HSA structure. The presence of the 50 mM of the Aspirin moderately inhibits the beta sheets formation of HSA in presence of glucose. Further, pre-acetylated HSA had evidently shown protection of the secondary structure by inhibiting the formation of beta sheets. Extent of AGE formation in terms of protein browning and carbonylation can be studied spectrometrically. HSA incubated with 0.5 M glucose

for 30 days showed the maximum absorbance at 340 nm, and the absorbance of control HSA remained constant throughout the incubation whereas in aspirin inhibitory assay showed less browning in compare with glycated HSA. As hypothesised, the pre-acetylated HSA glycation assay showed less browning. (Figure 4.3). Protein cross linking can be clearly visible by SDS-PAGE, where aggregates/cross-linkers can be seen as new bands above the normal protein because of the increment in mass and low protein mobility by AGE formation. SDS gel electrophoresis was performed for the matured HSA assay for aspirin inhibition and pre-acetylation (Figure 4.2). It was evident from the SDS-PAGE that glycation induces cross-linking in HSA and could not able to separate on the gel. Aspirin inhibition assay shows decrease in the crosslink formation as proved earlier (lane D) [29]. Here also pre-acetylated HSA incubated with glucose shows less crosslink formation in comparison with glycated HSA (Lane B) and aspirin inhibition (LaneD). Earlier studies were also shown the glycation inhibitory effects in different proteins like crystallin [18], collagen [30], haemoglobin [31], fibrinogen [32], HSA [29], etc. Further, we are able to show *In vivo* that pre-acetylation of proteins can protect them from the glycation reaction and AGE formations.

4.3.3 Effect on Diabetic Mice

This protective effect of pre-acetylation was demonstrated *in vivo* in STZ induced diabetic mice. Along with the glycation associated parameters such as glycated haemoglobin (HbA1c)(Fig. 4.4) and plasma AGEs levels, were monitored by western blotting (Fig. 4.4). STZ induced diabetes led to increase in HbA1c ($\geq$7.0 %) and plasma AGE levels, which was decreased significantly in pre-acetylated mice with aspirin (200 mg/L). Pre-acetylation of proteins was achieved by treating Balb/c mice for one month prior to STZ injection

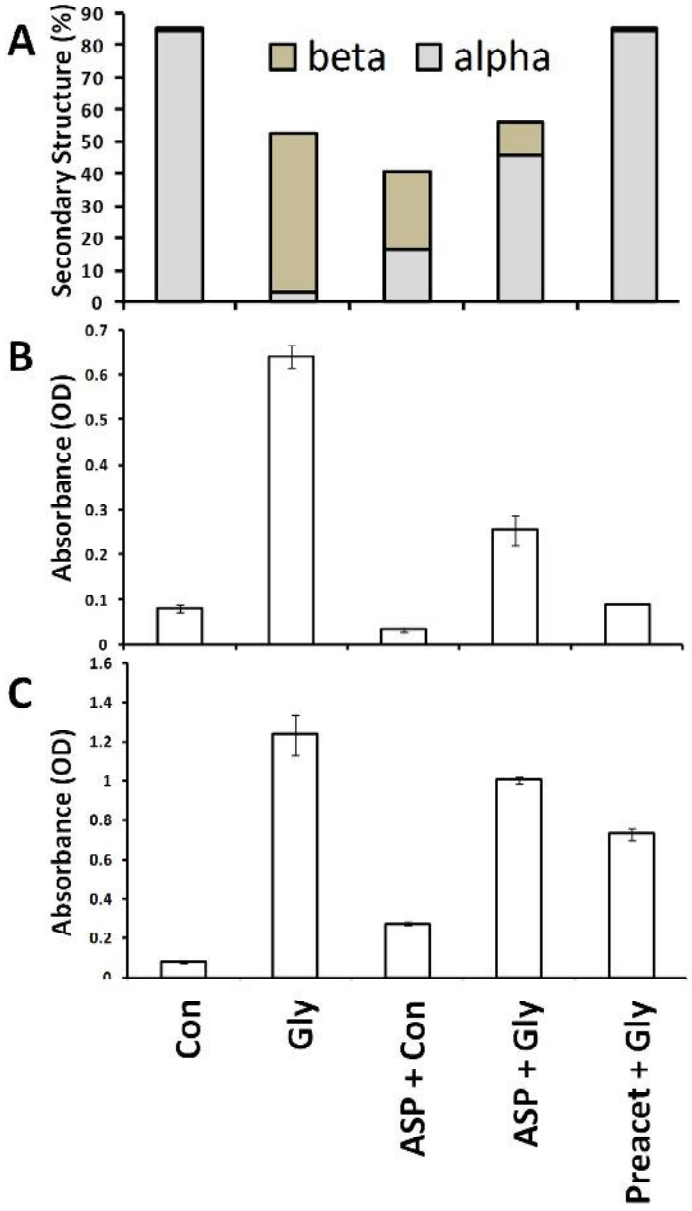

Figure 4.3: ***In vitro* HSA glycation Assay.** A) Glycation induced protein secondary structure differences observed by CD-Spectroscopy, B) Browning of protein estimated by UV- Vis spectroscopy at 340 nm C) Carbonylation of protein measured by UV- Vis spectroscopy at 365 nm

with aspirin and further the acetylation is confirmed by western blotting. Diabetic as well as pre-acetylated diabetic mice groups were simultaneously injected with STZ for the induction of diabetes and the treatment is continued further. To nullify the glycation inhibitory effects of aspirin, one of the normal STZ induced diabetic mice group are also treated with the same dosage of aspirin. The HbA1c are not increased with time, and was near to normal levels (6.4%) within 30 days in pre-acetylated mice. However, treatment of aspirin for 30 days, even at same concentration (200mg/L) failed to decrease HbA1c (10.14%). Plasma AGE levels measured by western blot analysis clearly shows the benefits of early usage of aspirin or pre-acetylation of proteins on prevention of AGE formation. Pre-acetylated mice showed beneficial effects in terms of decreased levels of plasma cholesterol, triglycerides, blood urea nitrogen, and creatinine levels, as compared to diabetic mice and aspirin treated diabetic mice (Table. 4.1). So this observation has immediate relevance to high risk diabetic prone individuals. Clinically it was shown that low dose aspirin treatment can benefit cardiovascular and atherothrombosis but not in diabetes [35, 33, 36]. Until know it was considered that the benefits of aspirin treatment is associated with the blockage of thromboxane synthesis by acetylating platelet cyclo-oxygenase [34], but we hypothesized that a major global level, proteome wide acetylating activity of aspirin can protect proteins from deleterious effect of AGE formation and their by further complications. Aspirin is able to induce the diabetic control by altogether a different mechanism than exciting medication. Despite its proven acetylating activity and AGE protection efficacy, aspirin therapy is underutilized in patients with diabetes. Early usage of low dose aspirin is a viable opportunity for “high-risk” diabetic awaiting groups.

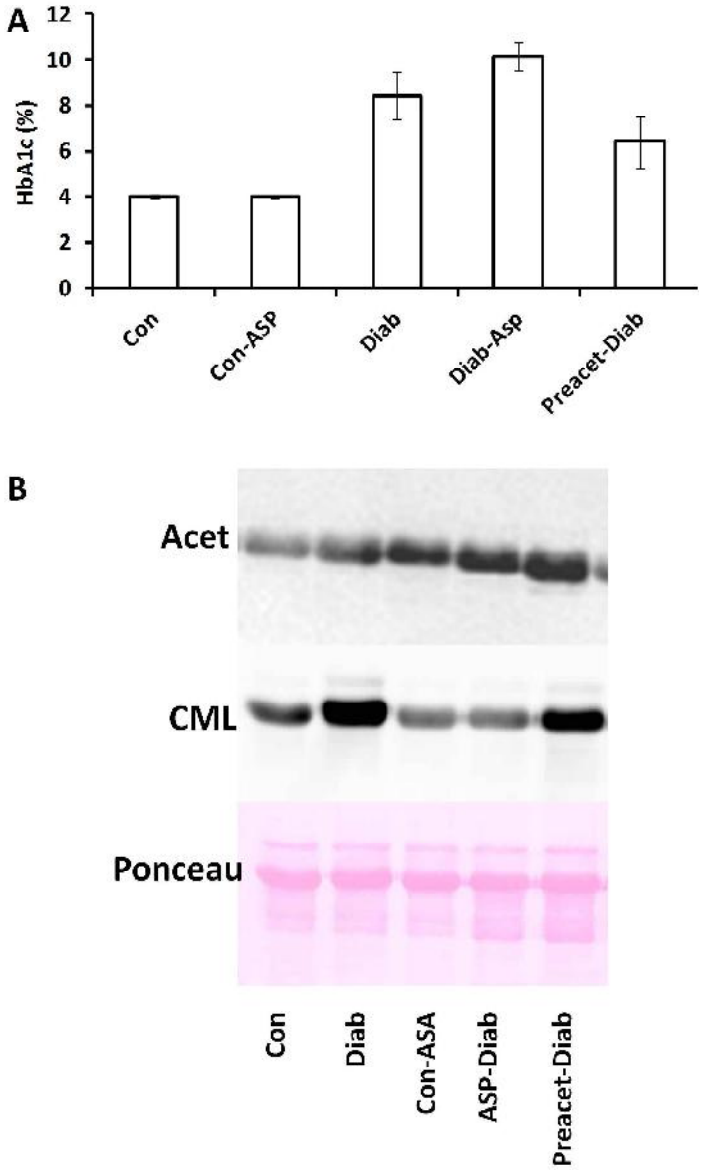

Figure 4.4: ***In vivo* glycation inhibition by pre-acetylation in diabetic mice.** A) Glycation in mice is monitored by measuring HbA1c. Pre-acetylated mice were able to show protection against glycation, as evidenced by low levels of HbA1c. B) Plasma protein glycation inhibition in STZ induced diabetic mice model. Plasma proteins were analysed for AGE modification by using antibodies against CML and acetylation is detected by using antiacetyl lysine antibody. The western blot shows lesser AGE levels in pre-acetylated mice plasma

4.4 Conclusion

In conclusion, pre-acetylation has showed a glycation protection activity and inhibits the further AGE modification which could form other complications. *In vitro* experiments with insulin and HSA have shown that it can prevent protein crosslink formation. Aspirin one of the multipurpose drug can do proteome level acetylation and protect AGE formation and the complications their after. *In vivo*, mice experiments further proved its efficacy. Clinically pre-acetylation of proteins for inhibition of AGE formation is minimally utilized. This study may emphasis on the need of a new prescription styles and new therapeutics with acetylating properties along with its other efficacy.

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Summary and Future Perspectives

Identifying safer drugs with multiple activities like Aspirin and Paracetamol is a dream for every drug discovery group. Many of the times the drug discovery pipeline fails in preclinical and clinical trials by unexpected drug toxicity, which is one of the major risks in the drug discovery processes. Thus drug discovery becomes very expensive and time consuming process, therefore, in this thesis, I have used drug repositioning approach, as the risk of failure is relatively lesser in this approach. A combination of chemical proteomics, comparative proteomics and disease specific (glycation inhibitor) approach was used for understanding the molecular mechanism of toxicity (e.g gatifloxacin), and for repositioning several drugs were screened for glycation inhibition activity to discover hydralazine as a transglycating agent. Furthermore, I have come up with a strategy to protect against glycation via pre-acetylation of proteins (aspirin). The results of the above experiments were summarized below.

Comparative and chemical proteomic studies were used to understand the mechanism of action of Gatifloxacin in *Saccharomyces cerevisiae.* Comparative proteomics was carried by two dimensional electrophoresis and mass spectrometry. The analysis suggests that the enzymes involved in glucose metabolism were deregulated by gatifloxacin. Chemical proteomic approach with gatifloxacin identified the interacting proteins as enolase, GAPDH, and Pyruvate Decarboxylase. Further, enolase interaction with gatifloxacin is confirmed by fluorescence quenching experiment. Both analyses suggest that

gatifloxacin affects glucose metabolism at different levels including gene expression, mRNA degradation, glucose transport and metabolism.

We also used target or disease based approach for high throughput screening. An anti-glycation assay was developed for identifying small molecules inhibitors of glycation, which will be helpful in treatment against diabetes. Hyperglycaemia in diabetes increases the AGE formation thus leads to different complications like neuropathy, nephropathy, retinopathy and other vascular diseases. Hence inhibition of glycation is an intervening strategy to ameliorate these complications. Here two different approaches are used to inhibit the glycation reaction. One is transglycation and the other one is pre-acetylation of proteins, these reactions either reverse the glycation or protect from glycation respectively.

In the first approach, small molecules were used to remove the bound glucose from schiff-base or amadori product called transglycation. A MALDI-TOF-MS based assay was developed for screening small molecules for transglycation activity. Hydralazine was identified as one of the transglycating agents. It was proved *in vitro* by Insulin and HSA based glycation inhibition assays. Additionally transglycation was demonstrated in streptozotocin induced diabetic mice at protein as well as by quantification of hydralazine glucose conjugate in the urine of diabetic mice treated with hydralazine. Glycation inhibition of proteins was showed as western blot of plasma and kidney proteins against various AGEs modification like CML, Pentosidine and AGE and by reduction in number of modified peptides of plasma abundant proteins by LC-MS^E approach. The glycation induced diabetic markers like Receptor for Advanced Glycation End products (RAGE), NADPH ox-

idase (NOX), and super oxide dismutase (SOD) were also down regulated in kidney. These findings will provide a new dimension for developing intervention strategies for the treatment of glycation associated diseases such as diabetes complications, atherosclerosis, and aging.

Aspirin mediated pre-acetylation has shown glycation protection activity and inhibits further AGE modification by *in vitro* experiments as well in *in vivo* in mice models. *In vitro* experiments were carried out with insulin and HSA as model proteins and shown that indeed pre-acetylation can prevent glycation induced protein carbonylation, browning, secondary structural change and crosslink formation. Additionally aspirin mediated pre-acetylation reduces HbA1c and AGE formation in the Streptozotocin induced diabetic mice. Hence pre-acetylating agents like aspirin have an additional therapeutic efficacy of reducing AGE levels. The identification of these novel acetylating agents represents a new area for drug discovery process.

Symposia and Conferences

Poster presentation / Participated

1. "*Proteome Wide Reduction in AGE Modification in STZ Induced Diabetic Mice by Hydralazine Mediated Transglycation* " International Conference on Mass Spectrometry,(ICMS-2013), M.G. University, Kottayam [2013].

2. "*Participated*" International symposium on "Proteomics Beyond IDs", CSIR-NCL, Pune [2012].

3. "*Understanding The Molecular Mechanism Of Gatifloxacin Induced Dysglycemic Effects By Chemical Proteomic Approaches* "$2^n d$ International Symposium on: Mass Spectrometry in Life Sciences, NCBS, Banglore [2011].

4. "*Participated*" ISMAS Syposium cum Workshop on Mass Spectrometry, BARC, Mumbai [2008].

5. "*Comparative and Chemical Proteomic Approaches Reveal Gatifloxacin Deregulates Enzymes Involved in Glucose Metabolism*" National Science Day Poster Presentation, NCL Research Foundation, National Chemical Laboratory, Pune, [2009].

List of Publications

1. "Proteome wide reduction in AGE modification in streptozotocin induced diabetic mice by hydralazine mediated transglycation": **Suresh K.K.,** Bhat, S., Golegaonkar, S.B., et al. *Sci. Rep.* 3 (2013) 2541.

2. ""Zoom ln"–A targeted database search for identification of glycation modifications analyzed by untargeted tandem mass spectrometry.": 2. Bhonsle, H.S., Korwar, A.M., **Suresh, K.K.,** Bhosale, S.D., Bansode, S.B. and Kulkarni, M.J. *Eur J Mass Spectrom (Chichester, Eng)* 18 (2012) 475.

3. "Comparative and chemical proteomic approaches reveal gatifloxacin deregulates enzymes involved in glucose metabolism.": **Suresh, K.K.,** Bhosale, S.D., Thulasiram, H.V. and Kulkarni, M.J. *J Toxicol Sci* 36 (2011) 787.

Awards and Recognition:

1. "**Award of Junior Research Fellowship (JRF)**" for 2007-2012, by Council of Scientific and Industrial Research (CSIR), Govt. of India, New Delhi.

2. "**Best poster award in Life Sciece**", National Science Day Celebration - 2009, CSIR-NCL, Pune